EXPRESS MAIL LABEL NO. EV077493871US

JC07 Rec'd PCT/PTO 18 MAR 2002

Form PTO-1390 (REV 11-98)	U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE	ATTORNEY'S DOCKET NUMBER 410718.90395
TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371		US. APPLICATION NO. (if known, see 37 CFR 1.5) 10/088405
INTERNATIONAL APPLICATION NO. PCT/CA00/01132	INTERNATIONAL FILING DATE 21 Sept 2000 (21.09.00)	PRIORITY DATE CLAIMED 21 Sept 1999 (21.09.99)

TITLE OF INVENTION LOCAL DELIVERY OF 17-BETA ESTRADIOL FOR PREVENTING VASCULAR INTIMA HYPERPLASIA AND FOR IMPROVING VASCULAR ENDOTHELIUM FUNCTION AFTER VASCULAR INJURY

APPLICANT(S) FOR DO/EO/US CHANDRASEKAR, Baskaran; TANGUAY, Jean-Francois

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. [X] This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.

2. [ ] This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.

3. [ ] This express request to begin national examination procedures (35 U.S.C. 371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and PCT Articles 22 and 39(1).

4. [X] A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.

5. [X] A copy of the International Application as filed (35 U.S.C. 371(c)(2))
   a. [ ] is transmitted herewith (required only if not transmitted by the International Bureau).
   b. [X] has been transmitted by the International Bureau.
   c. [ ] is not required, as the application was filed in the United States Receiving Office (RO/US)

6. [ ] A translation of the International Application into English (35 U.S.C. 371(c)(2)).

7. [X] Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3))
   a. [ ] are transmitted herewith (required only if not transmitted by the International Bureau).
   b. [X] have been transmitted by the International Bureau.
   c. [ ] have not been made; however, the time limit for making such amendments has NOT expired.
   d. [ ] have not been made and will not be made.

8. [ ] A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).

9. [ ] An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)).

10. [] A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).

Items 11. to 16. below concern document(s) or information included:

11. [ ] An Information Disclosure Statement under 37 CFR 1.97 and 1.98 and Form 1449.

12. [ ] An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.

13. [X] A FIRST preliminary amendment.
    [ ] A SECOND or SUBSEQUENT preliminary amendment.

14. [ ] A substitute specification.

15. [ ] A change of power of attorney and/or address letter.

16. [X] Other items or information: Copy of Form PCT/IB/308 dated 29 March 2001 Confirming Transmittal of the International Application to the US as Designated Office; Postcard

U.S. APPLICATION NO. (If known, see 37 C.F.R. 1.5) 10/088405	INTERNATIONAL APPLICATION NO. PCT/CA00/01132	ATTORNEY'S DOCKET NUMBER 410718.90395

17. [X] The following fees are submitted: **BASIC NATIONAL FEE (37 CFR 1.492(a)(1)-(5)):**	**CALCULATIONS** PTO USE ONLY	
Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO **$1040.00**		
International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO **$890.00**		
International preliminary examination fee (37 CFR 1.482) not paid to USPTO but international search fee (37 CFR 1.445(a)(2)) paid to USPTO **$740.00**		
International preliminary examination fee paid to USPTO (37 CFR 1.482) but all claims did not satisfy provisions of PCT Article 33(1)-(4) **$710.00**		
International preliminary examination fee paid to USPTO (37 CFR 1.482) and all claims satisfied provisions of PCT Article 33(1)-(4) **$100.00**		
ENTER APPROPRIATE BASIC FEE AMOUNT =	**$890.00**	
Surcharge of **$130.00** for furnishing the oath or declaration later than [ ]20 [ ]30 months from the earliest claimed priority date (37 CFR 1.492(e)).	**$**	

CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE		
Total claims	8 -20 =	0	X **$18.00**	**$**	
Independent claims	1 -3 =	0	X **$78.00**	**$**	
MULTIPLE DEPENDENT CLAIM(S) (if applicable)			+ **$260.00**	**$**	
TOTAL OF ABOVE CALCULATIONS =				**$890.00**	
[X] Applicant hereby claims small entity status. Reduction by 1/2 for filing by small entity.				$445.00	
SUBTOTAL =				**$445.00**	
Processing fee of **$130.00** for furnishing the English translation later than [ ]20 [ ]30 months from the earliest claimed priority date (37 CFR 1.429(f)). +				**$**	
TOTAL NATIONAL FEE =				**$445.00**	
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). **$40.00** per property +				**$**	
TOTAL FEES ENCLOSED =				**$445.00**	
				Amount to be: refunded	**$**
				Charged	**$**

a. [ ] A check in the amount of $ .00 to cover the above fees is enclosed.

b. [X] Please charge my Deposit Account No. 17-0055 in the amount of $445.00 to cover the above fees. A duplicate copy of this sheet is enclosed.

c. [X] The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. 17-0055. A duplicate copy of this sheet is enclosed.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO:

Quarles & Brady LLP
411 East Wisconsin Ave.
Milwaukee, WI 53202-4497

Jean C. Baker
SIGNATURE

Jean C. Baker
NAME

35,433
REGISTRATION NUMBER

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of:	**CHANDRASEKAR**	Docket No.:	410718.90395
Serial No.:	**Unassigned**	Filed:	**Concurrently herewith**
Int'l appln No.:	**PCT/CA00/01132**	Int'l filing date:	**21 Sept 2000**
Title:	**LOCAL DELIVERY OF 17-BETA ESTRADIOL FOR PREVENTING VASCULAR INTIMA HYPERPLASIA AND FOR IMPROVING VASCULAR ENDOTHELIUM FUNCTION AFTER VASCULAR INJURY**		

****

PRELIMINARY AMENDMENT

Box PCT
Assistant Commissioner for Patents
Washington, D.C. 20231

Dear Sir:

In connection with the above-identified application filed herewith, please enter the following preliminary amendment:

IN THE CLAIMS:

The claims have been amended to read as follows. A copy of the marked claims showing the amendments made is attached.

1. The use of 17-β estradiol or a derivative thereof in the making of a medication or a device for in-situ administration in the lumen of a blood vessel having suffered vascular injury, at the injured site, for improving reendothelization and vascular endothelial function in a patient.

2. The use as defined in claim 1, wherein 17-β estradiol or a derivative thereof is present in a dose unit of 1 to 5000 μp/Kg of patient's body weight.

3. The use, as defined in claim 1, wherein 17-β estradiol or a derivative thereof is present in a dose unit of 10 to 50 μp/Kg of patient's body weight.

4. The use as defined in claim 1, wherein 17-β estradiol or a derivative thereof is present in a dose unit of 10 to 30 μp/Kg of patient's body weight.

5. The use as defined in claim 1, wherein said pharmaceutically acceptable carrier comprises hydroxypropyl-beta-cyclodextrin (HPCD).

6. The use as defined in claim 5, wherein HPCD is present in a dose capable of solubilizing 17-beta estradiol or a derivative thereof.

7. The use as defined in claim 4, wherein 17-beta-estradiol or a derivative thereof is admixed with a carrier comprising at least 0.63 mg hydroxypropyl-beta-cyclodextrin per kilogram of patient's body weight.

8. The use as defined in claim 1, which is for a single administration.

Remarks

The above amendments are being made to eliminate multiple dependencies in the claims of this application.

No fee is believed necessary to enter this amendment. However if a fee is necessary, please charge Deposit Account 17-0055.

Applicant respectfully requests that the preliminary amendment described herein be entered into the record prior to examination and consideration of the above-identified application.

QUARLES & BRADY LLP

BY: Jean C. Baker
Jean C. Baker, Reg. No. 35,433

Date: March 18, 2002

QUARLES & BRADY
411 East Wisconsin Avenue
Milwaukee WI 53202-4497
U.S.A.
(414) 277-5709

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of:	**CHANDRASEKARr**	Docket No.:	410718.90395
Serial No.:	**Unassigned**	Filed:	**Concurrently herewith**
Int'l appln No.:	**PCT/CA00/01132**	Int'l filing date:	**21 Sept 2000**
Title:	**LOCAL DELIVERY OF 17-BETA ESTRADIOL FOR PREVENTING VASCULAR INTIMA HYPERPLASIA AND FOR IMPROVING VASCULAR ENDOTHELIUM FUNCTION AFTER VASCULAR INJURY**		

****

CLAIM SET SHOWING AMENDMENTS MADE

1. The use of 17-β estradiol or a derivative thereof in the making of a medication or a device for in-situ administration in the lumen of a blood vessel having suffered vascular injury, at the injured site, for improving reendothelization and vascular endothelial function in a patient.

2. The use as defined in claim 1, wherein 17-β estradiol or a derivative thereof is present in a dose unit of 1 to 5000 µp/Kg of patient's body weight.

3. The use, as defined in claim 1, wherein 17-β estradiol or a derivative thereof is present in a dose unit of 10 to 50 µp/Kg of patient's body weight.

4. The use as defined in claim 1, wherein 17-β estradiol or a derivative thereof is present in a dose unit of 10 to 30 µp/Kg of patient's body weight.

5. The use as defined in [any one of claims 1 to 5] claim 1, wherein said pharmaceutically acceptable carrier comprises hydroxypropyl-beta-cyclodextrin (HPCD).

6. The use as defined in claim 5, wherein HPCD is present in a dose capable of solubilizing 17-beta estradiol or a derivative thereof.

7. The use as defined in claim 4, wherein 17-beta-estradiol or a derivative thereof is admixed with a carrier comprising at least 0.63 mg hydroxypropyl-beta-cyclodextrin per kilogram of patient's body weight.

8. The use as defined in [any one of claims 1 to 7] claim 1, which is for a single administration.

TITLE OF THE INVENTION

Local Delivery of 17-beta Estradiol for Preventing vascular intimal hyperplasia and for improving vascular endothelium function after vascular injury

FIELD OF THE INVENTION

The present invention relates to the local use of estradiol or a derivative thereof to improve the outcome of a coronary angioplasty. More specifically, the present invention is concerned with the local use of estradiol or a derivative thereof for decreasing neointima hyperplasia that occurs during restenosis, and for improving the endothelium function after vascular injury, both events contributing to the ultimate success of an angioplasty.

BACKGROUND OF THE INVENTION

Restenosis is currently the major limitation of percutaneous transluminal coronary angioplasty (PTCA), and is seen in up to 30-40 % of patients.[1] The most important mechanisms contributing to restenosis are neointima proliferation, vascular remodelling, and elastic recoil.[2] Elastic recoil and vascular remodelling can be reduced to a large extent by stenting.[3] Although radiation therapy has been reported to show beneficial effeets,[4,5] no effective therapy exists yet for neointima proliferation. Vascular smooth muscle cell (SMC) migration and proliferation have been documented to occur as early as 36 hours following arterial injury.[6] In cell culture assays, 17-beta estradiol inhibited migration and proliferation of rat vascular SMC.[7,8] Similar effects have also been shown with human vascular SMC from saphenous vein.[9] Prolonged systemic administration of estrogen has been shown to inhibit intima hyperplasia in animal studies.[10,11] Instead of

administrating estradiol systematically we here tested how a local administration of 17-beta estradiol during PTCA could effectively inhibit neointima proliferation.

The vital role of endothelium in the regulation of vascular tone of arteries is well-recognized (1). The intact endothelium also has important inhibitory effects on platelet aggregation, monocyte adhesion, and vascular smooth muscle cell proliferation (2). Endothelial injury associated with endothelial dysfunction is known to occur as a consequence of percutaneous transluminal coronary angioplasty (PTCA) (3), and may play an important role in restenosis following PTCA (4). Impaired endothelial function has been demonstrated in porcine coronary arteries as long as 4 weeks following PTCA in pigs (5). Systemically administered 17-beta estradiol has been reported to accelerate endothelial recovery after arterial injury (10). Since endothelial injury due to PTCA is a local event, we hypothesized that local delivery of 17-beta estradiol following PTCA may enhance endothelial recovery.

SUMMARY OF THE INVENTION

An object of the present invention is therefore to provide efficient methods by which 17-β estradiol or a derivative thereof is used locally during PTCA to improve endothelial function after vascular injury and/or to decrease the neointima hyperplasia and/or prevent restenosis. Compositions for executing these methods are also a further object of this invention.

Other objects, advantages and features of the present invention will become more apparent upon reading of the following nonrestrictive description of preferred embodiments thereof, given by way of examples only, with reference to the accompanying drawings.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 Representative light micrographs (x 40 magnification) of arterial segments from the same animal, stained with Verhoeff's stain. 17-beta estradiol (a) treated segment shows markedly less neointima hyperplasia compared to PTCA only (b), or vehicle alone (c) groups. The extent of injury is similar in all 3 segments.

Figure 2 Comparison of (A) neointima area, (B) neointima/media area, (C) restenotic index, and (D) % stenosis between PTCA alone vs vehicle only, and PTCA only vs 17-beta estradiol groups; * $p < 0.05$, ** $p < 0,01$ *** $p < 0.002$. Values are expressed as mean ± SEM.

Figure 3 Representative coronary angiograms demonstrating the vasoconstrictive response to intracoronary infusion of acetylcholine (Ach) 10^{-4}M obtained from the same animal at 4 weeks following percutaneous transluminal coronary angioplasty (PTCA). Column A = basal, column B = after Ach, column C = following intracoronary nitroglycerin. Top panel = treatment with vehicle, mid panel = PTCA only, lower panel 17-beta estradiol treatment groups respectively.

Figure 4 Representative light micrographs (x 1000) of cross sections of vessels obtained from the same animal for immunohistochemical staining with the lectin *Dolichos biflorus* agglutinin (evident as dark brown staining of luminal surface). Vessels treated with 17-beta estradiol (A) demonstrate reendothelialization to a greater degree as compared to PTCA only (B) and vehicle (C) groups.

Figure 5 Representative light micrographs (x 1000) of cross sections of vessels obtained from the same animal, for immunohistochemical analysis

of endothelial nitric oxide synthase (eNOS) expression. Vessels treated with 17-beta estradiol (A) show greater expression of eNOS (evident as dark brown staining of luminal surface) as compared to PTCA only (B) and vehicle (C) groups.

Figure 6 Graph depicting correlation between vasoconstrictive response to Ach 10^{-4} M and (A) reendothelialization ($r = -0.48$, $p < 0.02$), (B) eNOS expression ($r = -0.58$, $p < 0.005$). Note: % vasoconstriction denotes % decrease in diameter following Ach 10^{-4} M as compared to the basal diameter.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

Example 1: The effect of estradiol on neointima hyperplasia

Methods

Animal preparation

Eighteen juvenile farm pigs (9 female, and 9 castrated male) weighing 20-25 kg were studied. The study was approved by, and conducted in accordance with, the guidelines of the Animal Care and Ethical Research Committee of the Montreal Heart Institute. Before the procedure, animals were given 650 mg of acetylsalicylic acid and 30 mg of nifedipine orally, premedicated with intramuscular injection of 6 mg/kg of a mixture of tiletamine hydrochloride and zolazepam hydrochloride, and given 0.05 mg of atropine. The invasive procedure was performed under general anesthesia with a mixture of isoflurane (1 to 1.5 %) and oxygen enriched air. The right femoral artery was cannulated percutaneously, and an 8 Fr arterial sheath was introduced. After arterial access had been obtained, 100 mg of lidocaine and 250 U/kg of heparin were administered intra-arterially via the sheath. Activated

coagulation time was maintained at > 300 seconds throughout the procedure.

Preparation of estradiol formulation

Each dose individually administered to the tested animals is composed of at least 12.5 mg hydroxypropyl-beta-cyclodextrin (HPCD) and 600 µg estradiol in a 5 ml solution volume.

A smaller or larger dose may be used. Indeed, the tested dose corresponds to the dose of about 675 µg formulated in a sublingual pellet and administered to postmenopausal women.[45] Such a dose may be unnecessarily high if administered locally. Indeed, doses of 200 and 400 µg have been tried and they were found to be as performing as the dose of 600 µg. Further, the necessary dose for performing the present invention may be influenced by the hormonal balance of the individual to be treated. Species variance is also a factor affecting the dosage regimen. Also, any derivative of 17-beta estradiol may replace the latter. A derivative is intended to cover a precursor, an active metabolite, an active analog or a modulator capable of positively influencing the activity of the receptor(s) to estradiol or of enhancing the binding and/or the activity of estradiol towards its receptor(s). Such derivatives are considered functional equivalents of 17-beta-estradiol, and therefore within the scope of this invention. A unit dose of 1 to 5000 µg/Kg of 17-beta-estradiol or an equivalent derivative dose is within the scope of this invention, preferably 10-50 µg/Kg, even more preferably 10-30 µg/Kg.

Angioplasty and Local Delivery

Standard PTCA equipment was used. An 8 Fr right Amplatz guiding catheter and right Judkins guiding catheter were used for cannulation of the left and right coronary arteries, respectively. PTCA was performed with a balloon size chosen to correspond to a balloon/artery ratio of 1.1-1.3. Three 30-second inflations at 10 atm pressure were performed with a 30-second interval between each inflation. Inflations were performed adjacent to major side branches to facilitate identification during harvesting, taking precaution not to include any side branch in the intended PTCA site. The left anterior descending, left circumflex, and right coronary arteries of each animal were subjected to PTCA. After PTCA, each coronary artery of an animal was randomized to receive either 600 µg of 17-beta estradiol locally, or vehicle alone locally, or PTCA only. The chemicals 17-beta estradiol and its vehicle 2-hydroxypropyl-beta-cyclodextrin (HPCD) were purchased from Sigma Chemical Co. The InfusaSleeve catheter (Local Med, Inc.) was used for local delivery.[12] Five ml of the designated substance was delivered at a driving pressure of 10 atm and support balloon pressure of 6 atm.

Of the 18 animals, 2 died a few days after PTCA, and were excluded; thus, 16 animals were analyzed. Twelve animals were euthanized at 28 days, and 4 at 7 days. After premedication and anesthesia, the right internal jugular vein and common carotid artery were cannulated. Following cross-clamping of the descending thoracic aorta exposed via a left lateral thoracotomy, exsanguination was performed, with simultaneous administration of 1 l of 0.9 % NaCl solution. The heart was perfusion-fixed in vivo with 2 l of 10 % buffered formalin at 200 mm Hg pressure, removed from the animal, and placed in 10 % buffered formalin solution. Coronary arteries were then dissected free from surrounding tissues. The site of PTCA was identified in relation to adjacent side branches, which served as landmarks. The injured

segment was harvested with a 1 cm normal segment proximal and distal to the injured site. Serial sections 3 to 5 mm long were made from the harvested segment, with a minimum of at least 3 sections (maximum 5) from each PTCA site. Sections were stored in buffered 10 % formalin and subjected to dehydration with increasing concentrations of alcohol, followed by treatment with xylene and paraffin. Each section was then cut to slices of 6 µm thickness with a microtome (Olympus cut 4060 E), and stained with Verhoeff's stain for morphometric analysis.

Morphometric analysis

Measurements were made with a video microscope (Leitz Diaplan, equipped with a Sony DXC 970 MD color video camera) linked to a 486 personal computer and customized software. A minimum of 3 sections for each injured segment were analyzed and results averaged. Analyses were made by a single observer unaware of the treatment group to which each segment had bee allocated. Randomly selected sections were viewed by a second observer (also blinded to protocol) independently; inter-observer variability was < 5 %. The areas of external elastic lamina (EEL), internal elastic lamina (IEL), and lumen were measured by digital planimetry; neointima (I) area (IEL - lumen area) and media (M) area (EEL - IEL area) were obtained. The % neointima was defined as the % of total vessel area occupied by neointima (% neointima = [I/EEL] x 100). Morphologic % stenosis was calculated as 100 (1 – lumen/IEL area).[13] The restenotic index was defined as [I/(I + M)]/(F/IEL circumference), where F is the fracture length of internal elastic lamina.[14] Histologic injury score was determined as previously defined.[15]

Immunohistochemistry

Following slicing with a microtome and blocking of non-specific antibodies, the sections were treated with mouse anti-proliferating cell nuclear antigen (PCNA) antibodies and diluted biotinylated goat anti-mouse antibodies. They were then incubated with avidin-biotin (Elite ABC Kit, Vector Laboratories), and developed with 3, 3'-diaminobenzidine (Vector Laboratories). They were finally counter-stained with hematoxylin. Porcine liver cells were used as a positive control. For each section, a 6 µm slice counter-stained with hematoxylin without treatment with the primary antibody (mouse anti- PCNA) served as a negative control.

The proliferative response to injury was studied by immunohistochemical analysis of samples from animals euthanized at 7 days. The % proliferating SMC was obtained by dividing the number of PCNA - positive SMC by the total number of SMC in each field; separate measurements were made for neointima and media layers. The proliferating cells were identified as SMC by positive staining of parallel sections with a smooth muscle actin antibody. To standardize comparison among treatment groups, measurements were obtained at 4 fixed locations separated by 90° sites for each section, and the results averaged. For each segment, two sections demonstrating maximal neointima response were analyzed, and the results averaged.

Statistical Analysis

Values are expressed as mean t standard deviation, except as otherwise indicated. Kruskal - Wallis analysis was used for comparison of data among the 3 groups; subsequently, 17-beta estradiol and vehicle alone groups were separately compared with the PTCA only group using the Mann - Whitney rank sum test. Chi - square analysis was used for comparison of proportions.

The Mann - Whitney rank sum test was also used for comparison of data between male and female animals within the 17-beta estradiol treated group. Values were considered statistically significant if $p < 0.05$.

Results

Following PTCA and local delivery, animals were allowed to recover, and gained weight steadily. Two animals died 48 and 72 hours after procedure respectively, and were not included; thus 16 animals were studied. Autopsy of the 2 animals revealed occlusive thrombus at the site of PTCA (in the 17-beta estradiol treated vessel in one pig, and in the vessel treated with PTCA only in the other pig).

Injured segments

Balloon/artery ratio and artery diameter were not significantly different among the 3 treatment groups (Table 1). Segments with intact IEL in which discernible injury was absent were excluded from analysis (2 from PTCA only group, and 1 from vehicle alone group). Two segments were lost during harvesting and processing (1 of vehicle alone, and 1 of PTCA only group).

Morphometric analysis

Of the 12 animals that underwent morphometric analysis at 28 days, arterial segments treated with local delivery of 17-beta estradiol showed significantly less neointima hyperplasia (Figure 1). This beneficial effect was noted in all parameters of neointima response to injury that were analyzed (Table 1). Of note, the extent of morphologic injury was similar among the 3 groups, suggesting that the use of the InfusaSleeve catheter was not associated with an enhanced risk of injury.

It was important to exclude an inhibitory effect on intima proliferation due to the vehicle, and, to confirm that the effect noted was in response to treatment with 17-beta estradiol. Analyses comparing segments treated with vehicle alone and PTCA only showed a similar response in terms of the extent of neointima proliferation. On the other hand, significantly less intima hyperplasia was observed in 17-beta estradiol treated segments as compared to segments treated with PTCA only (Figure 2). Compared to PTCA only, or vehicle alone, 17-beta estradiol decreased neointima formation by 54.6 % and 64.9 % respectively.

To exclude the possibility of influence of sex on response to estrogen, the 7 segments obtained from male pigs treated with 17-beta estradiol, and 5 segments obtained from female pigs treated with 17-beta estradiol were analyzed. No statistically significant differences were evident (Table 2).

Immunohistochemistry

The number of PCNA - positive SMC was low overall; sacrifice at an earlier time might have yielded a higher number. However, a statistically significant decrease in the proliferative response was seen in animals treated with 17-beta estradiol. Among the different groups, the % of PCNA - positive SMC in the neointima were 0.43 ± 0.52 % in 17-beta estradiol, 4.26 ± 2.33 % in PTCA only, and 4.27 ± 2.73 % in vehicle alone groups respectively ($p < 0.05$ for 17-beta estradiol vs other 2 groups). There were no statistically significant differences in % PCNA - positive SMC in the media among the 3 groups: 0.4 ± 0.3 %, 1.38 ± 1.74 %, and 1.24 ± 1.57 % for 17-beta estradiol, PTCA only, and vehicle alone groups respectively (p = NS).

Vascular remodeling

To determine the effect on vascular remodeling of the agents used, the EEL area of the injured segment and of the normal vessel proximal to site of PTCA were obtained, and their ratio calculated.[13] No significant difference among the groups was noted: 1.01 ± 0.16, 1.16 ± 0.28, 1.31 ± 0.37 respectively for 17-beta estradiol, PTCA only, and vehicle alone groups respectively (p = NS).

Conclusions

The present study demonstrates, for the first time, that locally delivered 17-beta estradiol decreases neointima proliferation following PTCA in pigs. The study also shows that the InfusaSleeve catheter can be used to deliver effectively 17-beta estradiol intramurally in coronary arteries.

Several previous experiments in animals have demonstrated that estrogen administered subcutaneously for up to 3 weeks inhibited the myointima response to arterial injury.[10,11] Recently, short-term subcutaneous estrogen therapy (6 to 17 days) was also shown to be effective in reducing the injury response in rat carotid artery.[16] Estrogen administered intra-muscularly for at least 3 weeks has also demonstrated the potential to inhibit vascular smooth muscle cell proliferation and neointima hyperplasia in rabbits.[17] However, the efficacy of local delivery of 17-beta estradiol to inhibit intima hyperplasia has not been previously studied.

The biologic effects of estrogen, like other steroid hormones, involve intracellular receptors. The first estrogen receptor (ER) to be discovered was ERα,[18,19] which was thought to mediate the beneficial effects of estrogen following vascular injury. ERα was also present in coronary arteries obtained

from autopsy specimens in both pre and postmenopausal women,[20] and in cell cultures of human saphenous vein and internal mammary artery specimens.[21] Recently, a second estrogen receptor, ERβ, has been identified in animals and humans.[22,23] The role of ERβ in response to vascular injury was subsequently demonstrated in experiments with ERα deficient mice.[24] Normal and ERα deficient mice treated with estrogen, when subjected to arterial injury, showed the same extent of inhibition of neointima proliferation compared to control mice; thereby demonstrating that inhibition of vascular injury response by estrogen is independent of ERα. Although the present experiment was not designed to study the mechanism of action of 17-beta estradiol, evidence exists for multiple potential mechanisms by which 17-beta estradiol can inhibit the vascular response to injury. Of importance may be the effect of 17-beta estradiol on nitric oxide (NO) synthesis. In cell culture studies with human and bovine endothelial cells, treatment with 17-beta estradiol stimulated NO synthase and increased NO production.[25,26] Postmenopausal women treated with transdermal 17-beta estradiol showed enhanced in vivo NO synthesis.[27] NO has demonstrated inhibitory effects on both migration [28] and proliferation [29] of vascular SMC, and decreased neointima formation after PTCA.[13] Preliminary reports have shown that therapy with 17-beta estradiol decreases intercellular and vascular cell adhesion molecule expression by human coronary SMC.[30] Cellular adhesion molecules are expressed by SMC following arterial injury[31] and their suppression with the use of monoclonal antibodies inhibited intima hyperplasia after arterial injury in rats.[32] The regulatory effect of 17-beta estradiol on vascular endothelial growth factor expression may also be partly responsible.[33-35] Perhaps the most important mechanism may be a direct inhibitory effect of 17-beta estradiol on vascular SMC proliferation.[36] The binding of 17-beta estradiol to its intracellular receptor activates DNA containing "estrogen responsive elements", leading to altered gene

expression. 17-beta estradiol also reduces platelet derived growth factor-induced migration and proliferation of vascular SMC.[9]

The beneficial effects of 17-beta estradiol, the predominant circulating estrogen in premenopausal women, on vascular injury response may not be replicated by other kinds of estrogens; for example, conjugated equine estrogen was found to have no effect on neointima proliferation in non-human primate models.[37] Simultaneous administration of progesterone may attenuate the vascular injury response to 17-beta estradiol.[38] A sexually dimorphic response to estrogen in intact rats has been reported following arterial injury, with male rats deriving no benefit with estrogen therapy .[39] This sexually dimorphic effect was, however, not observed in another experiment with gonadectomized rats.[11] In the present study, too, no significant difference in neointima proliferative response to 17-beta estradiol was noted between the sexes. Increased expression of ERβ mRNA (ERβ is directly associated with inhibition of vascular SMC proliferation) following arterial injury has been demonstrated in intact male rats;[40] of additional interest in the study is that no increase in ERα was seen following arterial injury.

17-beta estradiol is a lipophilic compound with poor solubility in aqueous solutions, thereby needing a vehicle for parenteral administration. HPCD is a starch derivative that has been successfully tested as an effective excipient for protein drugs.[41] The pharmacokinetics of HPCD are similar to that of inulin, and the toxic dose (nephrotoxicity) has been estimated to be 200 mg/kg in rats.[42] The dose of HPCD used to dissolve 17-beta estradiol in the present study was 0.63 mg/kg, far below the toxic dose. Furthermore, HPCD has been used for administration of ophthalmic preparations and intravenous anaesthetic agents in humans.[43,44] HPCD complexed to 17-beta estradiol has been used to enhance bioavailability of orally, or, sublingually administered 17-beta estradiol with no untoward effects in humans.[45]

Retrospective studies in humans have shown no benefit of hormonal replacement therapy on angiographic restenosis following PTCA[46] although one study did show a beneficial effect after directional atherectomy.[47] However, it should be noted that conjugated estrogen (and not 17-beta estradiol) was the predominant form of estrogen used in many of these patients, and, no information about concomitant use of progesterone is available.

In conclusion, we have shown that, a single dose of 17-beta estradiol delivered locally during PTCA has the potential to inhibit neointima proliferation effectively. The delivery of 17-beta estradiol can be performed easily with the InfusaSleeve catheter, without risk of additional injury. With this approach, it may be possible to avoid potential undesirable effects of long term systemic administration of estrogen. ERβ has been identified in humans, and inhibition of proliferation of human vascular SMC by 17-beta estradiol has been demonstrated in cell culture assays. The local administration of 17-beta estradiol is therefore a promising new approach, which might be useful in preventing the proliferative response after PTCA in humans. Its usefulness in preventing restenosis after PTCA is contemplative in view of the foregoing promising results.

Example 2: The effect of estradiol on vascular endothelial function

Methods

Animal preparation

The study protocol was approved by the Animal Care and Ethical Research Committee of the Montreal Heart Institute. Juvenile farm pigs weighing 20-25 kg (1 female, and 8 castrated males) were used. On the day of the experiment, animals received 650 mg of acetylsalicylic acid and 30 mg of

nifedipine orally, were premedicated with 6mg/kg of tiletamine hydrochloride and zolazepam hydrochloride, and were given 0.05 mg of atropine intramuscularly. Under general anesthesia (a mixture of 1-1.5% isoflurane and oxygen enriched air), the right femoral artery was cannulated percutaneously. An 8 Fr arterial sheath was introduced, and 100 mg/kg of lidocaine and 250 U/kg of heparin were administered intra-arterially. Additional heparin was administered during PTCA if needed, to maintain an activated coagulation time of > 300 seconds.

Procedure

An 8 Fr right Amplatz guiding catheter and right Judkins guiding catheter were used for cannulation of the left and right coronary arteries, respectively. A standard balloon catheter (corresponding to a balloon/artery ratio of 1.1-1.3: 1) was advanced over a 0.014" floppy guide wire, and 3 successive 30-second inflations at 10 atm pressure were made with a 30 second interval between each inflation. PTCA was performed on all 3 coronary meries of each animal. For local delivery, the InfusaSleeve catheter (LocalMed Inc.) was used, which permits safe drug delivery with negligible additional injury (7). After balloon dilatation, each coronary artery of an animal was randomized to receive either 600 µg of 17-beta estradiol (in 5 ml), vehicle alone (5 ml), or PTCA only. The vehicle 2-hydroxypropyl-beta-cyclodextrin (HPCD), and 17-beta estradiol were obtained from Sigma Chem. Co. For local delivery with the InfusaSleeve catheter, a proximal driving pressure of 10 atm and support balloon pressure of 6 atm were utilized.

Intracoronary infusion

All 9 animals underwent cardiac catheterization at the end of 4 weeks. After a baseline coronary angiogram, selective cannulation of the proximal portion

of a coronary artery was performed with a single lumen balloon catheter (TotalCross, Schneider) for the administration of vasoactive agents. Acetylcholine (Ach) in increasing concentrations of 10^{-7} M, 10^{-6} M, 10^{-5} M, 10^{-4} M, was successively infused through the lumen port of the catheter. Each dose was administered for a duration of 3 minutes at a constant rate of 1 ml/min using an infusion pump. Coronary angiography was performed at the end of each dose. After infusion of the highest concentration of Ach (10^{-4} M and angiography, 100 µg of nitroglycerin was administered via the lumen port of the catheter, and a coronary angiogram performed. The same protocol was repeated for the other 2 coronary arteries. Heart rate, blood pressure, and ECG were monitored continuously throughout the experiment.

Quantitative coronary angiography

Coronary angiography was performed with a single plane imaging system (Electromed Intl). Images were obtained in predetermined views which best demonstrated the vessel segment of interest and without overlap of branches. Care was taken to maintain the same angulation during angiography of a segment throughout the procedure. Ionic contrast (MD-76, Mallinckrodt Medical Inc) was used throughout the experiment. Images were captured at a frame speed of 30 frames/sec, and stored digitally. A segment of contrast-filled guiding catheter was included in every frame, for the purpose of calibration. Calibration was performed using the known diameter of the contrast-filled guiding catheter as the reference segment, to avoid error due to magnification. Coronary artery diameter measurements were made using a validated computerized edge-detection system (8). The midpoint of the injured segment was used for calculation of coronary artery diameter. For each analysis, coronary artery diameter measurements were performed in 3 consecutive end-diastolic frames, and the results averaged.

Measurements were performed by an independent observer blinded to the treatment group of the vessels.

Immunohistochemistry

The animals were euthanized at 4 weeks. Under general anesthesia as described above, exsanguination was performed with replacement by 1 l of 0.9 % NaCl solution. The heart was perfusion-fixed in vivo with 2 l of 10 % buffered formalin at 200 mm Hg pressure. The heart was then removed, and the coronary arteries were harvested immediately. From the injured segment (identified in relation to side branches), serial sections of 3-5 mm were made, and stored in 10 % buffered formalin solution. The sections were then treated with incremental concentrations of alcohol followed by treatment with xylene and paraffin. Slices of 6 µm thickness were prepared, and stained with Verhoeff's stain for assessment of tissue response to injury. For each injured segment, 2 slices demonstrating maximal neointima response were selected for immunohistochemistry, and the results obtained from analysis of the cross sections were averaged. The % of reendothelialization and, the % of endothelial nitric oxide synthase (eNOS) expression were calculated as follows: (the total length of the luminal surface staining positively / the perimeter of the lumen) x 100, respectively. Analysis was performed by an independent examiner with no knowledge of the treatment groups to which the sections belonged. For lectin immunohistochemistry, the 6 µm slices were first treated with hydrogen peroxide and methanol to block endogenous peroxide, incubated with the *Dolichos biflorus* agglutinin (Sigma Chemical Co.) followed by treatment with 3,3'-diaminobenzidine (Vector Laboratories) and, subsequently counter-stained with hematoxylin. For immunohistochemistry of eNOS expression, after blocking of endogenous peroxide and non-specific antibodies, the slices were treated serially with the primary mouse anti-eNOS antibody (Bio/Can Scientific), the secondary goat anti-

mouse antibody (Vector Laboratories), incubated with avidin-biotin (Vector Laboratories), treated with 3,3'-diaminobenzidine (Vector Laboratories) and finally counter-stained with hematoxylin. For both immunohistochemical examinations, normal porcine carotid artery slices were used as positive controls; whereas slices obtained from the injured coronary arteries and stained only with hematoxylin were used as negative controls.

Statistical analysis

Values are expressed as mean ± SD. Comparison of basal coronary artery diameter among the 3 groups was made using the one-way analysis of variance test. Comparisons between basal coronary artery diameter and coronary artery diameter following infusion of vasoactive agents were made with two-tailed Student's t-tests. The Kruskal-Wallis test was used for comparison of lectin and eNOS expression among the 3 treatment groups. Linear relationships between lectin expression and response to Ach, and between eNOS expression and response to Ach were analyzed with Pearson correlation coefficients. Values were considered to be statistically significant if $p < 0.05$.

Results

There were no significant differences in basal coronary artery diameter (2.53 ± 0.6 mm for 17-beta estradiol, 2.79 ± 0.35 mm for PTCA only, and 2.77 ± 0.44 mm for vehicle groups respectively, $p < 0.4$) among the 3 treatment groups. The extent of morphologic tissue injury (9) among the groups was similar. No changes in heart rate, ECG, or blood pressure were noted during the local delivery or during intracoronary infusion of vasoactive agents.

Response of PTCA only group to Ach

Compared to the basal coronary artery diameter, there were no significant changes in coronary artery diameter following intracoronary infusion of 10^{-7} M and 10^{-6} M concentrations of Ach (Table). At a concentration of 10^{-4} M a significant vasoconstrictive response was noted ($p < 0.02$). A marked vasoconstrictive response was observed at a concentration of 10^{-4} M ($p < 0.0001$) (Figure 3). The vasoconstriction was completely reversed upon administration of the endothelium-independent vasodilator nitroglycerin. Coronary diameter increased from 1.8 ± 0.48 mm after 10^{-4} M Ach, to 2.5 ± 0.28 mm following nitroglycerin ($p < 0.01$; $p = 0.2$ for post-nitroglycerin vs basal diameter).

Response of vehicle treatment group to Ach

Compared to the basal coronary artery diameter, 10^{-7} M Ach did not change coronary artery diameter in the vehicle treatment group (Table 3). A trend towards significant vasoconstriction was noted with 10^{-6} M Ach ($p = 0.06$). Significant vasoconstriction was produced by 10^{-5} M ($p < 0.02$), and at 10^{-4} M ($p < 0.001$) Ach infusion respectively (Figure 3). Nitroglycerin completely reversed the vasoconstriction, returning the arteries to their basal diameter (from 1.89 ± 0.51 mm after 10^{-4} M Ach, to 2.69 ± 0.52 mm following nitroglycerin [$p < 0.004$; $p = 0.7$ for post-nitroglycerin vs basal diameter]).

Response of 17-beta estradiol treated group to Ach

In the vessels treated with local delivery of 17-beta estradiol no significant vasoconstrictive response to Ach occurred at any concentration used (Table) (Figure 3). A mild and statistically nonsignificant increase in coronary artery diameter was observed following administration of nitroglycerin: from 2.28 ±

0.61 mm after 10^{-4} M Ach to 2.61 ± 0.48 mm after nitroglycerin ($p = 0.4$; $p = 0.8$ for post-nitroglycerin vs basal diameter).

Immunohistochemistry

Immunohistochemical analyses were performed 4 weeks after PTCA on all 9 animals. Three arterial segments were lost/damaged during harvesting of the samples (2 of PTCA only group, and I of vehicle group). Significant differences were seen among the 3 treatment groups in the extent of reendothelialization, as assessed by immunohistochemical analysis with the lectin *Dolichos biflorus* agglutinin (Figure 4). Reendothelialization was noted to a greater extent in vessels treated with local delivery of 17-beta estradiol compared to the other 2 groups (90.6 ± 5.5 % for 17-beta estradiol 71 ± 6.8 % for PTCA only, and 72.8 ± 4.9 % for vehicle, $p < 0.0005$). Endothelial nitric oxide synthase expression was also higher in vessels treated with 17-beta estradiol (35.6 ± 11.8 % for 17-beta estradiol 9.4 ± 3.9 % for PTCA only, and 9.2 ± 4.0 % for vehicle, $p < 0.0005$) (Figure 5). No significant differences in immunohistochemical analyses were observed between vessels treated with vehicle or PTCA only.

We proceeded further to analyze whether a linear relationship between reendothelialization and the response to Ach could be demonstrated. A significant inverse correlation was noted between reendothelialization as assessed by immunohistochemistry with the lectin *Dolichos biflorus* agglutinin and the response to Ach ($r = 0.48$, $p < 0.02$) (Figure 6). An even stronger inverse linear correlation was observed between eNOS expression and the response to Ach ($r = -0.58$, $p < 0.005$).

Conclusions

This study demonstrates for the first time that local delivery of 17-beta estradiol immediately following PTCA enhances subsequent reendothelialization and endothelial function at the site of injury. Besides its critical role in the regulation of vascular tone, the normal endothelium functions as an effective barrier between blood elements and underlying vascular smooth muscle cells. Endothelium-derived nitric oxide (NO) is a potent vasodilator, inhibits monocyte adherence and platelet aggregation and adhesion (10), vascular smooth muscle cell migration (11) and proliferation (12).

PTCA is associated with arterial injury and damage to the endothelium (3). Following arterial injury, varying rates of reendothelialization have been reported. Reendothelialization rates of 81 % (13), and even lower rates of < 50 % (14) following arterial injury have been observed. In a study of specimens of restenotic lesions obtained by atherectomy in humans, no endothelial cells could be demonstrated (15). In the present study, local treatment with 17-beta estradiol was followed by nearly complete reendothelialization (90.6 ± 5.5 %), which was significantly greater than that observed in the groups not treated with 17-beta estradiol. Estrogen receptors have been identified in human coronary artery and umbilical vein endothelial cells (16), and when bound to estrogen are capable of regulating protein synthesis by altering transcription rates (17). In cell culture assay of human umbilical vein endothelial cells, treatment with 17-beta estradiol markedly increased both cell migration and proliferation (18). Therapy with subcutaneously implanted 17-beta estradiol pellets significantly enhanced reendothelialization following arterial injury (6). The capacity of 17-beta estradiol to increase vascular endothelial growth factor synthesis (19) and the effort of 17-beta estradiol on basic fibroblast growth factor may be

responsible for the enhanced reendothelialization. Vascular endothelial growth factor treatment is known to promote reendothelialization in vivo (20). In human umbilical vein and coronary artery endothelial cell culture experiments, treatment with 17-beta estradiol enhanced the release and phosphorylation of basic fibroblast growth factor (21,22). It has been shown that administration of basic fibroblast growth factor in vivo stimulates reendothelialization following arterial injury in rats (23). Another mechanism by which 17-beta estradiol could possibly influence extent of reendothelialization is by inhibition of apoptosis of injured endothelial cells: a 50 % decrease in apoptosis was seen with 17-beta estradiol treatment of human umbilical vein endothelial cells exposed to tumor necrosis factor-α (24). It is noteworthy that increased expression of tumor necrosis factors is known to occur following balloon injury (25)-

Impaired endothelial function, as in atherosclerosis (26) or following experimental inhibition of NO (27), has been associated with a paradoxical constrictive response to Ach. This paradoxical response to Ach could be modified by treatment with estrogen. In humans, 17-beta estradiol administered intravenously (28) or by continuous intracoronary infusion (29), attenuated the vasoconstrictive response to Ach and also inhibited the Ach-induced increase in coronary resistance and decrease in coronary blood flow. The regulatory effect of 17-beta estradiol on eNOS that we observed may be responsible for the beneficial effects on endothelial function, as vascular response to Ach is closely related to eNOS expression (30,31). In support of this notion, a strong inverse linear relationship was seen between the vascular response to Ach and eNOS expression (Figure 4). The ability of estrogen to induce nitric oxide synthase was first identified during gestation in guinea pigs (32). Induction of eNOS function by 17-beta estradiol has been subsequently demonstrated to be accompanied by increased eNOS protein and mRNA expression (33,34). Increased circulating NO levels

have been observed in postmenopausal women treated with 17-beta estradiol (35). Following arterial injury, the regenerated endothelium is often functionally abnormal (5). Abnormal vasomotion as a result of persistent endothelial dysfunction at the site of angioplasty has been demonstrated in patients undergoing PTCA, and is postulated to be responsible for the symptom of angina noted in patients with nonsignificant stenosis following PTCA (36). We have shown that functional abnormalities could be improved significantly by treatment with locally delivered 17-beta estradiol. A unifying hypothesis for the responses we observed is that eNOS downregulation following PTCA prevents the vasodilatory response to Ach mediated by endothelial NO production. By improving eNOS expression, 17-beta estradiol allows the vasodilatory response of Ach to counteract its direct vasoconstricting action, preventing Ach-induced vasoconstriction at the site of local injury. The vasodilatory response to nitroglycerin in Ach-constricted arteries post-PTCA is consistent with this concept, since exogenous nitroglycerin (which is a NO donor) simply provides a local NO-related dilation that the eNOS deficient angioplastied segment cannot provide for itself.

Both rapid non-genomic and genomic effects have been postulated to be involved in the influence of 17-beta estradiol on coronary vasculature (37,38). Although increased protein synthesis was not quantified in the present study, the enhanced eNOS expression and the response to Ach observed as late as 28 days following a single dose of 17-beta estradiol appears to be consistent with a genomic effect. This is the first study to suggest the existence of a genomic effect following local therapy with 17-beta estradiol in coronary circulation in vivo.

Gender differences in the endothelium-dependent vasodilation by 17-beta estradiol have been noted (39). In our study, a majority of animals were

mates and a significant beneficial effect of 17-beta estradiol was noted in all the animals studied, irrespective of sex. Thus, local delivery of 17-beta estradiol appears to be effective in males as well as females. There is evidence to suggest that the simultaneous administration of progesterone reduces NO levels induced by 17-beta estradiol (35), this issue was, however, beyond the scope of the present study.

We conclude that a single dose of 17-beta estradiol delivered locally following balloon injury can significantly improve reendothelialization and enhance endothelial function at the injured site as late as 1 month following injury. Besides the beneficial vascular effects of improved endothelial function, this observation may be of particular importance following balloon angioplasty as improved endothelial function is known to be associated with decreased neointima formation in the injured area (20,40). This approach merits further study, with a view to potential clinical value in the prevention of vascular dysfunction and restenosis following PTCA.

Formulations

The formulations may include estradiol or a derivative thereof and any pharmaceutically acceptable vehicle. Since estradiol is a lipophilic molecule, such vehicle would ideally include a solvent component. Such a solvent component includes molecules such as propylene glycol, ethanol, and detergents, for example Pluronics™. The formulations may take the form of a liquid, a suspension, a semi-solid or a thermoreversible composition which may form a layer over the endothelium. The formulations may further be included in or used as a coating for a device such as a stent, or be part of any similar device that can be left *in-situ* upon angioplasty or vascular surgery.

Although the present invention has been described hereinabove by way of preferred embodiments thereof, these embodiments can be modified at will, without departing from the spirit and nature of the subject invention. Such modifications are within the scope of the present invention as defined in the appended claims.

Table 1: Morphometric Analysis

Characteristics	17-beta estradiol	PTCA only	Vehicle alone	p value*
Segments analyzed	12	9	10	NS
Artery size (mm)	2.86 ± 0.35	2.94 ± 0.24	2.94 ± 0.41	NS
Balloon/Artery ratio	1.22 ± 0.09	1.2 ± 0.06	1.17 ± 0.11	NS
EEL_{ref}/EEL_{inj} †	1.01 ± 0.16	1.31 ± 0.37	1.16 ± 0.28	NS
Neointima area (mm^2)	0.4 ± 0.3	0.88 ± 0.61	1.14 ± 1.03	< 0.05
% neointima	12.16 ± 8.89	23.02 ± 11.91	25.46 ± 14.96	< 0.025
Neointima/Media area	0.59 ± 0.48	1.67 ± 1.29	1.75 ± 1.29	< 0.01
% stenosis	15.67 ± 11.13	27.51 ± 13.17	30.34 ± 17.05	< 0.025
Restenotic index	1.3 ± 0.5	2.4 ± 0.68	2.42 ± 0.71	< 0.005
Injury score	1.64 ± 0.34	1.7 ± 0.43	1.77 ± 0.47	NS

* 17-beta estradiol vs other 2 groups; †EEL_{ref} = proximal reference segment external elastic lamina area, EEL_{inj} = injured segment external elastic lamina area (averaged).

Table 2: Response to 17-beta estradiol According to Sex of the Animal

Characteristics	Male	Female	p value
Restenotic index	1.2 ± 0.59	1.37 ± 0.45	> 0.1
Neointima area (mm^2)	0.51 ± 0.34	0.25 ± 0.15	> 0.1
Neointima/Media area	0.78 ± 0.55	0.32 ± 0.16	> 0.1
% neointima	14.93 ± 10.68	8.29 ± 3.72	> 0.1
% stenosis	18.93 ± 13.39	11.09 ± 5.16	> 0. I

Table 3: Response to Intracoronary Acetylcholine

Ach*	Diameter-basal (mm)	Diameter-post Ach (mm)	p value
PTCA group			
10^{-7}M	2.79 ± 0.35	2.65 ± 0.35	0.4
10^{-6}M	2.79 ± 0.35	2.54 ± 0.32	0.1
10^{-5}M	2.79 ± 0.35	2.3 ± 0.35	0.02
10^{-4}M	2.79 ± 0.35	1.8 ± 0.48	0.0001
Vehicle group			
10^{-7}M	2.77 ± 0.44	2.6 ± 0.41	0.4
10^{-6}M	2.77 ± 0.44	2.33 ± 0.5	0.06
10^{-5}M	2.77 ± 0.44	2.24 ± 0.47	0.02
10^{-4}M	2.77 ± 0.44	1.89 ± 0.51	0.001
17-beta estradiol group			
10^{-7}M	2.53 ± 0.6	2.46 ± 0.58	0.8
10^{-6}M	2.53 ± 0.6	2.38 ± 0.58	0.6
10^{-5}M	2.53 ± 0.6	2.36 ± 0.59	0.6
10^{-4}M	2.53 ± 0.6	2.28 ± 0.61	0.4

* acetylcholine

References cited in Example 1

[1] Dangas G, Fuster V. Management of restenosis after coronary intervention. Am Heart J 1996 ;132 :428-36.

[2] Post MJ, Borst C, Kuntz RE. The relative importance of arterial remodelling compared with intima hyperplasia in lumen narrowing after balloon angioplasty. Circulation 1994; 89: 2816-21.

[3] Currier JW, Faxon DP. Restenosis after percutaneous transluminal coronary angioplasty: Have we been aiming at the wrong target? J Am Coll Cardiol 1995; 25: 516-20.

[4] Teirstein PS, Massullo V, Jani S, Popma JJ, Mintz GS, Russo RJ, Schatz RA, Guarneri EM, Steuterman S, Morris NB, Leon MB, Tripuraneni P. Catheter-based radiotherapy to inhibit restenosis after coronary stenting. N Engl J Med 1997; 336: 1697-703.

[5] King SBIII, Williams DO, Chougule P, Klein JL, Waksman R, Hilstead R, Macdonald J, Anderberg K, Crocker IR. Endovascular beta-radiation to reduce restenosis after coronary balloon angioplasty: results of the Beta Energy Restenosis Trial (BERT). Circulation 1998; 97: 2025-30.

[6] Clowes AW, Reidy MA, Clowes MM. Kinetics of cellular proliferation after arterial injury: smooth muscle cell growth in the absence of endothelium. Lab Invest 1983; 49: 327-3 3 .

[7] Akishita M, Ouchi Y, Miyoshi H, Kozaki K, Inoue S, Ishikawa M, Eto M, Toba K, Orimo H. Estrogen inhibits cuff- induced intima thickening of rat

femoral artery: effects on migration and proliferation of vascular smooth muscle cells. Atherosclerosis 1997; 130: 1-10.

[8] Kolodgie FD, Jacob A, Wilson PS, Carlson GC, Farb A, Verma A, Virmani R. Estradiol attenuates directed migration of vascular smooth muscle cells in vitro. Am J Pathol 1996; 148: 969-76.

[9] Dai - Do D, Espinosa E, Liu G, Rabelink TJ, Julmy F, Yang Z, Mahler F, Luscher TF. 17-beta estradiol inhibits proliferation and migration of human vascular smooth muscle cells: similar effects in cells from postmenopausal females and in males. Cardiovascular Research 1996; 32: 980-5.

[10] Sullivan Jr TR, Karas RH, Aronovitz M, Faller GT, Ziar JP, Smith JJ, O'Donnell Jr TF, Mendelsohn ME. Estrogen inhibits the response - to - injury in a mouse carotid artery model. J Clin Invest 1995; 96: 2482-8.

[11] Chen SJ, Li H, Durand J, Oparil S, Chen YF. Estrogen reduces myointima proliferation after balloon injury of rat carotid artery. Circulation 1996; 93: 577-84.

[12] Moura A, Lam JYT, Hebert D, Kermode JR, Grant GW, Robitaille D, Klein EJ, Yock PG, Simpson JB, Kaplan AV. Intramural delivery of agent via a novel drug - delivery sleeve: histological and functional evaluation. Circulation 1995; 92: 2299-2305.

[13] Varenne O, Pislaru S, Gillijns H, Pelt NV, Gerard RD, Zoldhelyi P, Van de Werf F, Collen D, Janssens S. Local adenovirus - mediated transfer of human endothelial nitric oxide synthase reduces luminal narrowing after coronary angioplasty in pigs. Circulation 1998; 98: 916-26.

[14] Bonan R, Paiement P, Scortichini D, Cloutier MJ, Leung TK. Coronary restenosis: evaluation of a restenosis injury index in a swine model. Am Heart J 1993; 126: 1334-40.

[15] Karas SP, Gravanis MB, Santoian EC, Robinson KA, Anderberg KA, King III SB. Coronary intima proliferation after balloon injury and stenting in swine: an animal model of restenosis. J Am Coll Cardiol 1992; 20: 467-74.

[16] Mori T, Durand J, Chen YF, Thompson JA, Oparil S. Short term estrogen treatment prior to and following balloon injury of rat carotid artery effectively blunts the vascular injury response. J Am Coll Cardiol 1999; 33 (2 suppl A): 259A (abstract).

[17] Foegh ML, Asotra S, Howell MH, Ramwell PW. Estradiol inhibition of arterial neointima hyperplasia after balloon injury. J Vasc Surg 1994; 19(4): 722-6.

[18] Colburn P, Buonassis V. Estrogen - binding sites in endothelial cell cultures. Science 1978; 201: 817-9.

[19] Venkov CD, Rankin AB, Vaughan DE. Identification of authentic estrogen receptor in cultured endothelial cells: a potential mechanism for steroid hormone regulation of endothelial function. Circulation 1996; 94: 727-33.

[20] Losordo DW, Kearney M, Kim EA, Jekanowski J, Isner JM. Variable expression of the estrogen receptor in normal and atherosclerotic coronary arteries of premenopausal women. Circulation 1994; 89: 1501-10.

[21] Karas RH, Patterson BL, Mendelsohn ME. Human vascular smooth muscle cells contain functional estrogen receptor. Circulation 1994; 89: 1943-50.

[22] Kuiper CiGMJ, Enmark E, Pelto - Huikko M, Nilsson S, Gustafsson JA. Cloning of a novel estrogen receptor expressed in rat prostrate and ovary. Proc Natl Acad Sci USA 1996; 93: 5925-5930.

[23] Mosselman S, Polman J, Dijkema R. ER/3: identification and characterization of a novel human estrogen receptor. FEBS Left 1996; 392: 49-53.

[24] Iafrati MD, Karas RH, Aronovitz M, Kim S, Sullivan Jr TR, Lubahn DB, O'Donnell Jr TF, Korach KS, Mendelsohn ME. Estrogen inhibits the vascular injury response in estrogen receptor a-deficient mice.

[25] Hishikawa K, Nakaki T, Marumo T, Suzuki H, Kato R, Saruta T. Up - regulation of nitric oxide synthase by estradiol in human aortic endothelial cells. FEBS Lett 1995; 360: 291-3.

[26] Hayashi T, Yamada K, Esaki T, Kuzuya M, Satake S, Ishikawa T, Hidaka H, Iguchi A. Estrogen increases endothelial nitric oxide by a receptor - mediated system. Biochem Biophys Res Commun 1995; 214(3): 847-55.

[27] Rosselli M, Imthurn B, Keller PJ, Jackson EK, Dubey RK. Circulating nitric oxide (nitrite/nitrate) levels in postmenopausal women substituted with 170-estradiol and norethisterone acetate: a two-year follow-up study. Hypertension 1995; 25(part 2): 848-53.

[28] Sarkar R, Meinberg EG, Stanley JC, Gordon D, Webb RC. Nitric oxide reversibly inhibits the migration of cultured vascular smooth muscle cells. Circ Res 1996; 78: 225-230.

[29] Cornwell TL, Arnold E, Boerth NJ, Lincoln TM. Inhibition of smooth muscle cell growth by nitric oxide and activation of cAMP-dependent protein kinase by cGMP. Am J Physiol 1994; 267: C1405-13.

[30] Speir E, Yu ZX, Ferrans VJ, Cannon III RO. Estrogen inhibits transcription factor and cell adhesion molecule activation in cytokine-stimulated human coronary smooth muscle cell via antioxidant effects. Circulation 1998; suppl I: I-220 (abstract).

[31] Tanaka H, Sukhova GK, Swanson SJ, Clinton SK, Ganz P, Cybulsky MI, Libby P. Sustained activation of vascular cells and leucocytes in the rabbit aorta after balloon injury. Circulation 1993; 88: 1788-1803.

[32] Yasukawa H, Imaizumi T, Matsuoka H, Nakashima A, Morimatsu M. Inhibition of intima hyperplasia after balloon injury by antibodies to intercellular adhesion molecule-1 and lymphocyte function - associated antigen-1. Circulation 1997; 95: 1515-22.

[33] Hyder SM, Stancel GM, Chiappetta C, Murthy L, Boettger-Tong HL, Makela S. Uterine expression of vascular endothelial growth factor is increased by estradiol and tamoxifen. Cancer Res 1996; 56(17): 3954-60.

[34] McLaren J, Prentice A, Charnock-Jones DS, Millican SA, Muller KH, Sharkey AM, Smith SK. Vascular endothelial growth factor is produced by peritoneal fluid macrophages in endometriosis and is regulated by ovarian steroids. J Clin Invest 1996; 98: 482-9.

[35] Asahara T, Bauters C, Pastore C, Kearney M, Rossow S, Bunting S, Ferrara N, Symes JF, Isner JM. Local delivery of vascular endothelial growth factor accelerates reendothelialization and attenuates intima hyperplasia in balloon-injured rat carotid artery. Circulation 1995; 91: 2793-2801.

[36] Mendelsohn ME, Karas RH. Estrogen and the blood vessel wall. Current Opinion in Cardiology 1994; 9: 619-26.

[37] Geary RL, Adams MR, Benjamin ME, Williams JK. Conjugated equine estrogens inhibit progression of atherosclerosis but have no effect an intima hyperplasia or arterial remodelling induced by balloon catheter injury in monkeys. J Am Coll Cardiol 1998; 31: 1158-64.

[38] Levine RL, Chen SJ, Durand J, Chen YF, Oparil S. Medroxyprogesterone attenuates estrogen-mediated inhibition of neointima formation after balloon injury of the rat carotid artery. Circulation 1996; 94: 2221-7.

[39] Oparil S, Levine RL, Chen SJ, Durand J, Chen YF. Sexually dimorphic response of the balloon-injured rat carotid artery to hormone treatment. Circulation 1997; 95: 1301-7.

[40] Lindner V, Kim SK, Karas RH, Kuiper GGJM, Gustafsson JA, Mendelsohn ME. Increased expression of estrogen receptor-f3 mRNA in male blood vessels after vascular injury. Circ Res 1998; 83: 224-9.

[41] Brewster ME, Hora MS, Simpkins JW, Bodor N. Use of 2-hydroxypropyl-betacyclodextrin as a solubilizing and stabilizing excipient for protein drugs. Pharm Res 1991; 8(6): 792-5.

[42] Frijlink HW, Visser J, Hefting NR, Oosting R, Meijer DKF, Lerk CF. The pharmacokinetics of beta-cyclodextrin and 2-hydroxypropyl-beta-cyclodextrin in the rat. Pharm Res 1990; 7(12): 1248-52.

[43] Kristinsson JK, Fridriksdottir H, Thorisdottir S, Sigurdardottir AM, Stefansson E, Loftsson T. Dexamethasone-cyclodextrin-polymer co-complexes in aqueous eye drops: aqueous humor pharmacokinetics in humans. Invest Ophthalmol Vis Sci 1996; 37: 1199-1203.

[44] Doenicke A, Roizen MF, Nebauer AE, Kugler A, Hoernecke R, Beger-Hintzen H. A comparison of two formulations for etomidate, 2-hydroxypropyl-beta-cyclodextrin (HPCD) and propylene glycol. Anesth Analg 1994; 79: 933-9.

[45] Hoon TJ, Dawood Y, Khan-Dawood FS, Ramos J, Batenhorst RL. Bioequivalence of a 17-beta estradiol hydroxypropyl-beta-cyclodextrin complex in postmenopausal women. J Clin Pharmacol 1993; 33: 1116-21.

[46] O'Keefe JH, Kim SC, Hall RR, Cochran VC, Lawhorn SL, McCallister BD. Estrogen replacement therapy after coronary angioplasty in women. J Am Coll Cardiol 1997; 29: 1-5.

[47] O'Brien JE, Peterson ED, Keeler GP, Berdan LG, Ohman EM, Faxon DP, Jacobs AK, Topol EJ, CaliffRM. Relation between estrogen replacement therapy and restenosis after percutaneous coronary interventions. J Am Coll Cardiol 1996; 28: 1111-8.

References cited in Example 2

[1] Furchgott RF, Zawadzki JV. The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine. Nature 1980; 288: 373-6.

[2] Rubanyi GM. The role of endothelium in cardiovascular homeostasis and diseases. J Cardiovasc Pharmacol 1993; 22(Suppl. 4): S1 -S 14.

[3] Fischell TA, Derby G, Tse TM Stadius ML. Coronary artery vasoconstriction routinely occurs after percutaneous transluminal coronary angioplasty: a quantitative arteriographic analysis. Circulation 1988; 78: 1323-34.

[4] Chesebro JH Lam JY, Badimon L, Fuster V. Restenosis after arterial angioplasty: a hemorrheologic response to injury. Am J Cardiol 1987; 60: 10B-16B.

[5] Shimokawa H Aarhus LL, Vanhoutte PM. Porcine coronary arteries with regenerated endothelium have a reduced endothelium-dependent responsiveness to aggregating platelets and serotonin. Cire Res 1987; 61: 256-70.

[6] Krasinski K, Spyridopoulos I, Asahara T, et al. Estradiol accelerates functional endothelial recovery after arterial injury. Circulation 1997; 1768-72.

[7] Moura A, Lam JYT, Hebert D, et al. Intramural delivery of agent via a novel drug- delivery sleeve: histologic and functional examination. Circulation 1995; 92: 2299- 2305.

[8] Mancini GBJ, Simon SB, McGillem MJ, et al. Automated quantitative coronary arteriography: morphologic and functional validation in vivo of a rapid digital angiographic method. Circulation 1987; 75(2): 452-60.

[9] Karas SP, Gravanis MB, Santoian EC, et al. Coronary intima proliferation after balloon injury and stenting in swine: an animal model of restenosis. J Am Coll Cardiol 1992; 20: 467-74.

[10] Cooke JP, Tsao PS. Cytoprotective effects of nitric oxide. Circulation 1993; 88(5): 2451-4.

[11] Sarkar R, Meinberg EG, Stanley JC, et al. Nitric oxide reversibly inhibits the migration of cultured vascular smooth muscle cells. Circ Res 1996; 78: 225-230.

[12] Cornwell TL, Arnold E, Boerth NJ, Lincoln TM. Inhibition of smooth muscle cell growth by nitric oxide and activation of cAMP-dependent protein kinase by cGMP. Am J Physiol 1994; 267: C1405-13.

[13] Hayashi Y, Tomoike H Nagasawa K., et al. Functional and anatomical recovery of endothelium H1090.

[14] Lindner V, Reidy MA Fingerie J. Regrowth of arterial endothelium: denudation with minimal trauma leads to complete endothelial cell growth. Lab Invest 1989; 61: 556- 63.

[15] Bauriedel G, Windstetter U, DeMario Jr SJ, et al. Migratory activity of human smooth muscle cells cultivated from coronary and peripheral

primary and restenotic lesions removed by percutaneous atherectomy. Circulation 1992; 85: 554-64.

[16] Kim-Schulze S, McGowan KA, Hubchak SC, et al. Expression of an estrogen receptor by human coronary artery and umbilical vein endothelial cells. Circulation 1996; 94: 1402-7.

[17] Venkov CD, Rankin AB, Vaughan DE. Identification of authentic estrogen receptor in cultured endothelial cells: a potential mechanism for steroid hormone regulation of endothelial function. Circulation 1996; 94: 727-33.

[18] Morales DE, McGowan KA, Grant DS, et al. Estrogen promotes angiogenic activity in human umbilical vein endothelial cells in vitro and in a murine model. Circulation 1995; 91: 755-63.after denudation of coronary artery. Am J Physiol 1988; 254: H1081-

[19] Hyder SM Stancel GM Chiappetta C, et al. Uterine expression of vascular endothelial growth factor is increased by estradiol and tamoxifen. Cancer Res 1996; 56(17):3964-60.

[20] Asahara T, Bauters C, Pastore C, et al. Local delivery of vascular endothelial growth factor accelerates reendothelialization and attenuates intima hyperplasia in balloon- injured rat carotid artery. Circulation 1995; 91: 2793-2801.

[21] Kim-Schulze S, Lowe WL, Schnapper HW. Estrogen stimulates delayed mitogen-activated protein kinase activity in human endothehal cells via an autocrine loop that involves basic fibroblast growth factor. Circulation 1998; 98: 413-21.

[22] Albuquerque ML, Akiyama SK, Schnaper HW. Basic fibroblast growth factor release by human coronary artery endothelial cells is enhanced by matrix proteins, 17-beta estradiol and a PKC signaling pathway. Exp Cell Res 1998; 245(1): 163-9.

[23] Lindner V, Majack RA, Reidy MA- Basic fibroblast growth factor stimulates endothelial regrowth and proliferation in denuded arteries. J Clin Invest 1990; 85: 2004-8.

[24] Spyridopoulos I Sullivan AB, Kearney M et al. Estrogen-receptor - mediated inhibition of human endothelial cell apoptosis: estradiol as a suraval factor. Circulation 1997; 95: 1505-14.

[25] Tanaka H Sukhova G, Schwartz D, Libby P. Proliferating arterial smooth muscle cells after balloon injury express TNF-α but not interleukin-1 or basic fibroblast growth factor. Arterioscier Thromb Vasc Biol 1996; 16: 12-18.

[26] Ludmer PL, Selwyn AP, Shook TL, et al. Paradoxical vasoconstriction induced by acetylcholine in atherosclerotic coronary arteries. N Eng J Med 1986; 315: 1046-5 1.

[27] Collins P, Burman J, Chung H Fox K. Hemoglobin inhibits endothelium-dependent relaxation to acetylcholine in human coronary arteries in vivo. Circulation 1993; 87: 80-5.

[28] Reis SE, Gloth ST, Blumenthal RS, et al. Ethinyl estradiol acutely attenuates abnormal coronary vasomotor responses to acetylcholine in postmenopausal women. Circulation 1994; 89: 52-60.

[29] Gilligan DM Quyyumi AA, Cannon III RO. Effects of physiological levels of estrogen on coronary vasomotor function in postmenopausal women. Circulation 1994; 89: 2545-51.

[30] Seo KK Yun HY, Kim H Kim SC. Involvement of endothelial nitric oxide synthase in the impaired endothelium-dependent relaxation of cavernous smooth muscle in hypercholesterolemic rabbit. J Androl 1999; 20(2): 298-306.

[31] Kullo IJ, Mozes G, Schwartz RS, et al. Enhanced endothelium-dependent relaxations after gene transfer of recombinant endothelial nitric oxide synthase to rabbit carotid arteries. Hypertension 1997; 30(part 1): 314-20.

[32] Weiner CP, Lizasoain I Baylis SA, et al. Induction of calcium-dependent nitric oxide syntheses by sex hormones. Proc Natl Acad Sci USA 1994; 91: 5212-16.

[33] Hishikawa K., Nakaki T, Marumo T, et al. Up-regulation of nitric oxide synthase by estradiol in human aortic endothelial cells. FEBS Letters 1995; 360: 291-3.

[34] MacRitchie AN, Jun SS, Chen Z, et al. Estrogen upregulates endothelial nitric oxide synthase gene expression in fetal pulmonary artery endothelium. Circ Res 1997; 81: 355-62.

[35] Rosselli M Imthum B, Keller PJ, et al. Circulating nitric oxide (nitrite/nitrate) level.% in postmenopausal women substituted with 17β-estadiol and norethisterone acetate: a two-year follow-up study. Hypertension 1995; 25(part 2): 848-53.

[36] Malekianpour M Doucet S, Lesperance J, et al. Abnormal coronary vasomotion and angina after successful coronary angioplasty. Circulation 1996; 94(suppl 1): I-560.

[37] Williams JK, Adams MR, Herrington DM, Clarkson TB. Short-term administration of estrogen and vascular responses of atherosclerotic coronary arteries. J Am Coll Cardio 1992; 20: 452-7.

[38] Wellman GC, Bonev AD, Nelson MT, Brayden JE. Gender differences in coronary artery diameter involve estrogen, nitric oxide, and Ca2+-dependent K+ channels. Circ Res 1996; 79: 1024-30.

[39] Kawano H, Motoyama T, Kugiyama K, et al. Gender differences in improvement of endothelium-dependent vasodilation after estrogen supplementation. J Am Coll Cardiol 1997; 30: 914-9.

[40] Chandrasekar B, Tanguay JF. Local delivery of 17-beta estradiol decreases neointima hyperplasia following coronary angioplasty in porcine model. (Submitted for publication).

WHAT IS CLAIMED IS:

1. The use of 17-β estradiol or a derivative thereof in the making of a medication or a device for in-situ administration in the lumen of a blood vessel having suffered vascular injury, at the injured site, for improving reendothelization and vascular endothelial function in a patient.

2. The use as defined in claim 1, wherein 17-β estradiol or a derivative thereof is present in a dose unit of 1 to 5000 µg/Kg of patient's body weight. ≈ 4 claims

3. The use, as defined in claim 1, wherein 17-β estradiol or a derivative thereof is present in a dose unit of 10 to 50 µg/Kg of patient's body weight. ≈ 5 claims

4. The use as defined in claim 1, wherein 17-β estradiol or a derivative thereof is present in a dose unit of 10 to 30 µg/Kg of patient's body weight. ≈ 6 claims

5. The use as defined in any one of claims 1 to ~~5~~ 4, wherein said pharmaceutically acceptable carrier comprises hydroxypropyl-beta-cyclodextrin (HPCD). ≈ 7 claims

6. The use as defined in claim 5, wherein HPCD is present in a dose capable of solubilizing 17-beta estradiol or a derivative thereof. ≈ 8 claims

7. The use as defined in Claim 4, where 17-beta-estradiol or a derivative thereof is admixed with a carrier comprising at

least 0.63 mg hydroxypropyl-beta-cyclodextrin per kilogram of patient's body weight.

8. The use as defined in any one of claims 1 to 7, which is for a single administration.

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
29 March 2001 (29.03.2001)

PCT

(10) International Publication Number
WO 01/21157 A2

(51) International Patent Classification[7]: A61K 31/00

(21) International Application Number: PCT/CA00/01132

(22) International Filing Date:
21 September 2000 (21.09.2000)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
2,282,982 21 September 1999 (21.09.1999) CA
2,300,246 9 March 2000 (09.03.2000) CA

(71) Applicant *(for all designated States except US)*: INSTITUT DE CARDIOLOGIE DE MONTREAL [CA/CA]; 5000 Bélanger Street East, Montreal, Quebec H1T 1C8 (CA).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: CHANDRASEKAR, Baskaran [IN/IN]; 2, Justice Ramanujam Road, Dr. Radhakrishnan Nagar, Chennai 600 041 (IN).

TANGUAY, Jean-François [CA/CA]; C.P. 323, succ. Mont-Royal, Monreal, Quebec H3P 3C5 (CA).

(74) Agents: DUBUC, Jean, H. et al.; Goudreau Gage Dubuc, The Stock Exchange Tower, Suite 3400, 800 Place Victoria, P.O. Box 242, Montreal, Quebec H4Z 1E9 (CA).

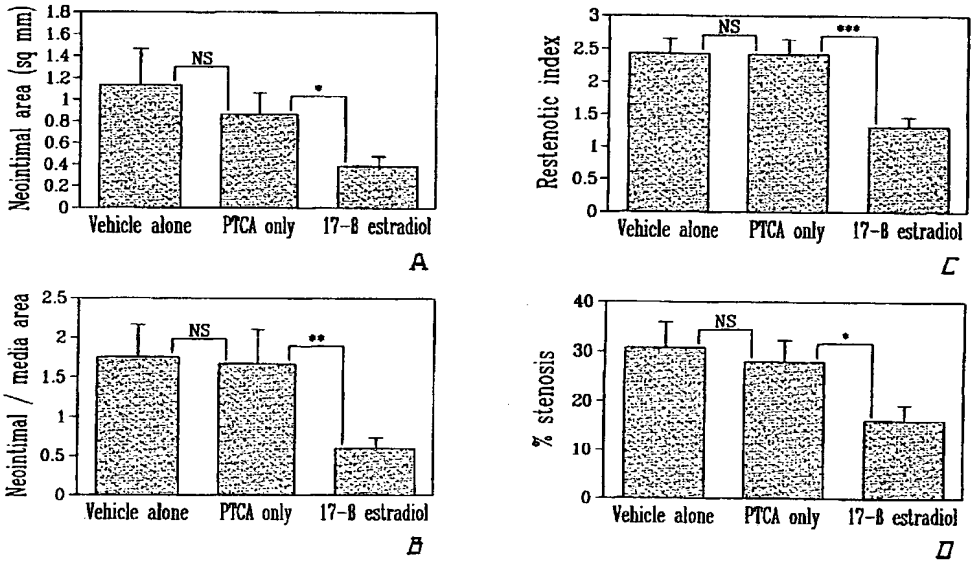
(81) Designated States *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CR, CU, CZ, DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.

(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published:
— *Without international search report and to be republished upon receipt of that report.*

[Continued on next page]

(54) Title: LOCAL DELIVERY OF 17-BETA ESTRADIOL FOR PREVENTING VASCULAR INTIMA HYPERPLASIA AND FOR IMPROVING VASCULAR ENDOTHELIUM FUNCTION AFTER VASCULAR INJURY



(57) Abstract: The cardioprotective effects of estrogen are well recognized. In *in vitro* experiments, and upon systemic administration, 17-beta estradiol has shown to inhibit vascular smooth muscle cell proliferation and intima hyperplasia and to improve vascular endothelium function, after vascular injury. We hypothesized that locally delivered 17-beta estradiol could prevent restenosis. Compositions are use of 17-beta estradiol for *in-situ* administration at a vascular injured site are objects of the present invention.

WO 01/21157 A2

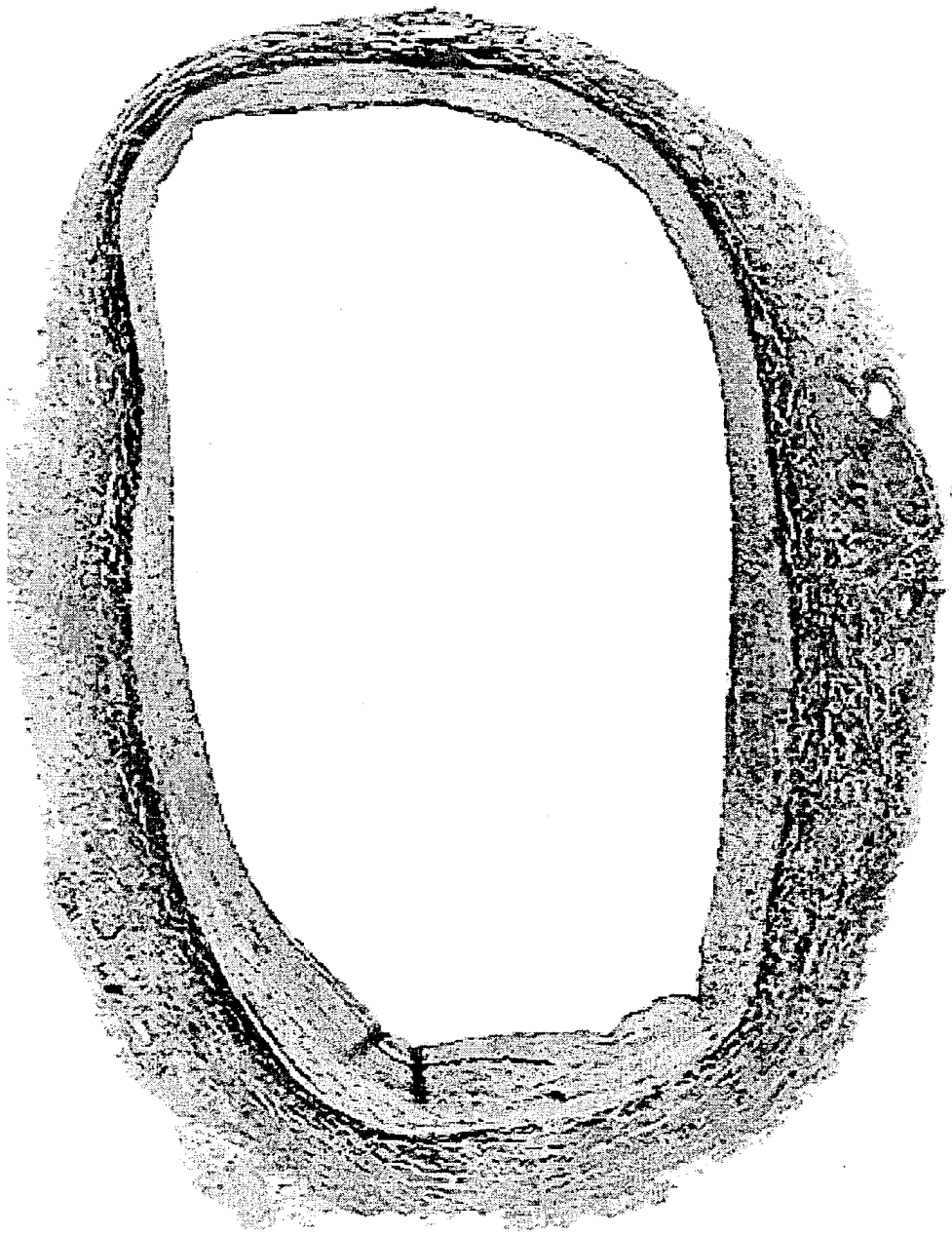


FIG. 1A

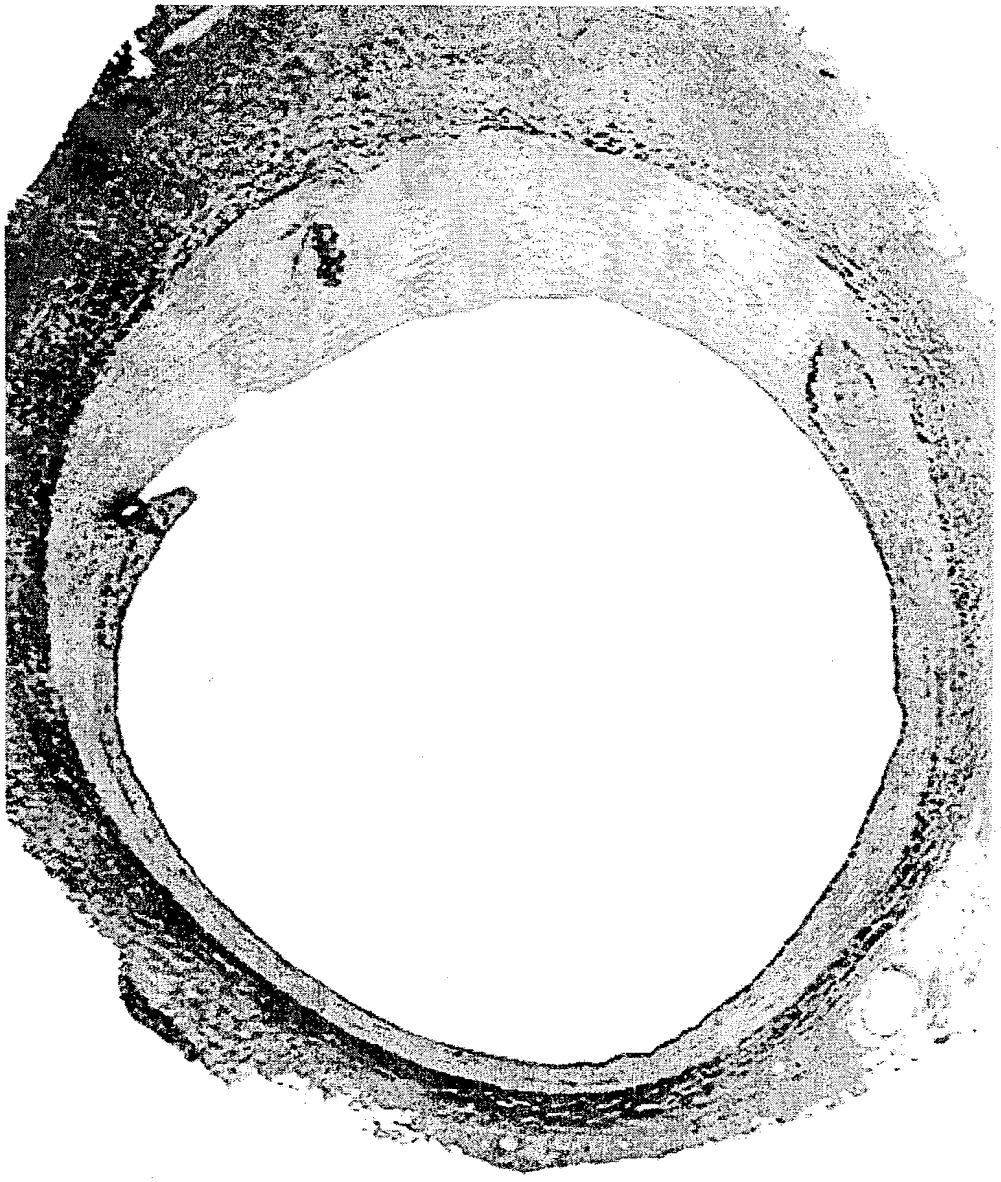


Fig. 1B

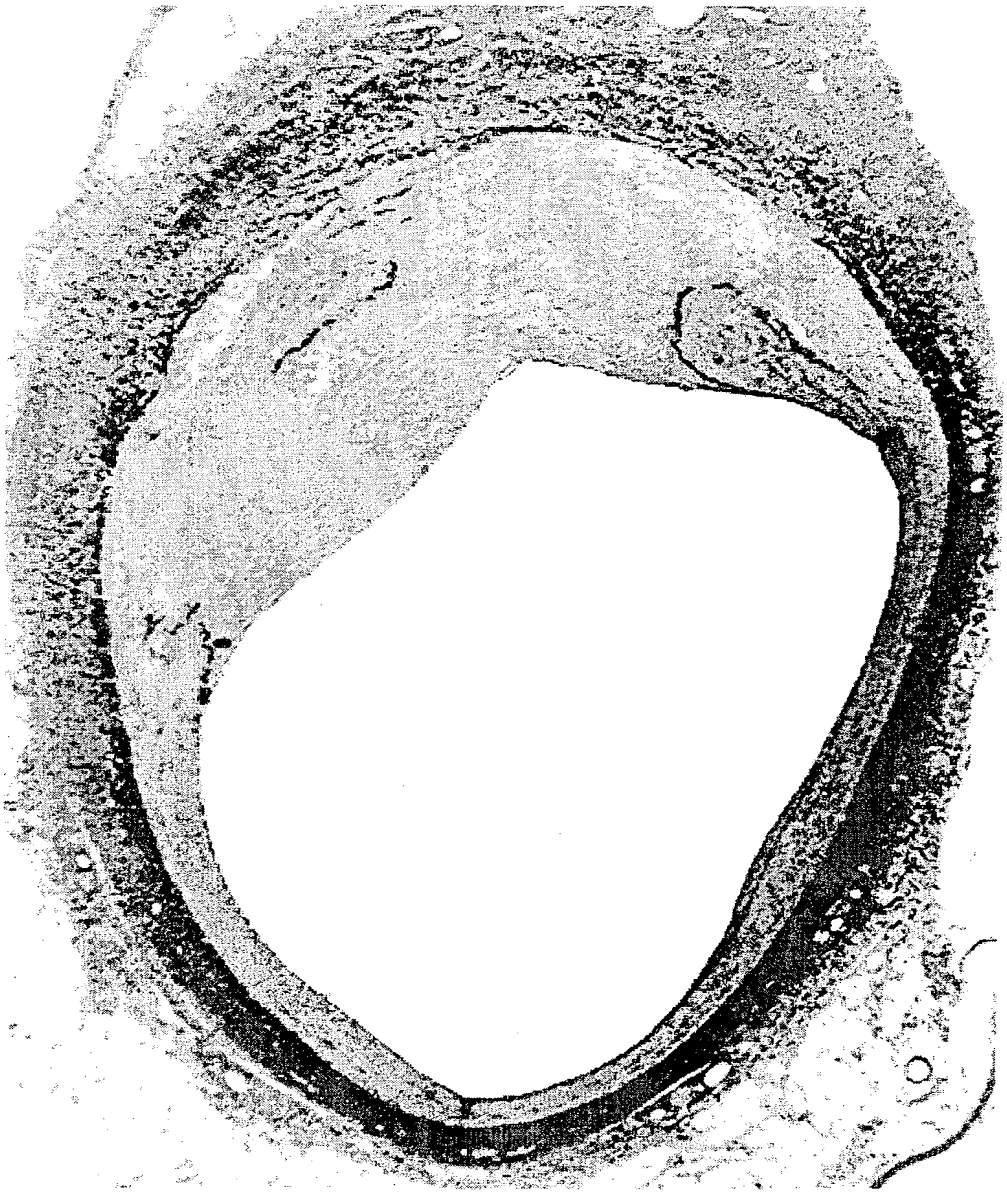


FIG-1C

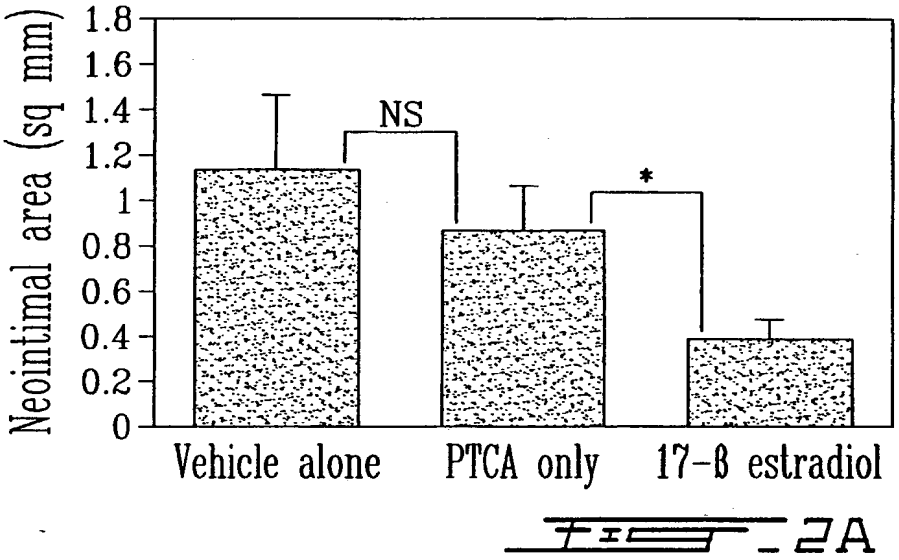

Neointimal area (sq mm)
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
NS
*
Vehicle alone
PTCA only
17-ß estradiol

Fig 2A

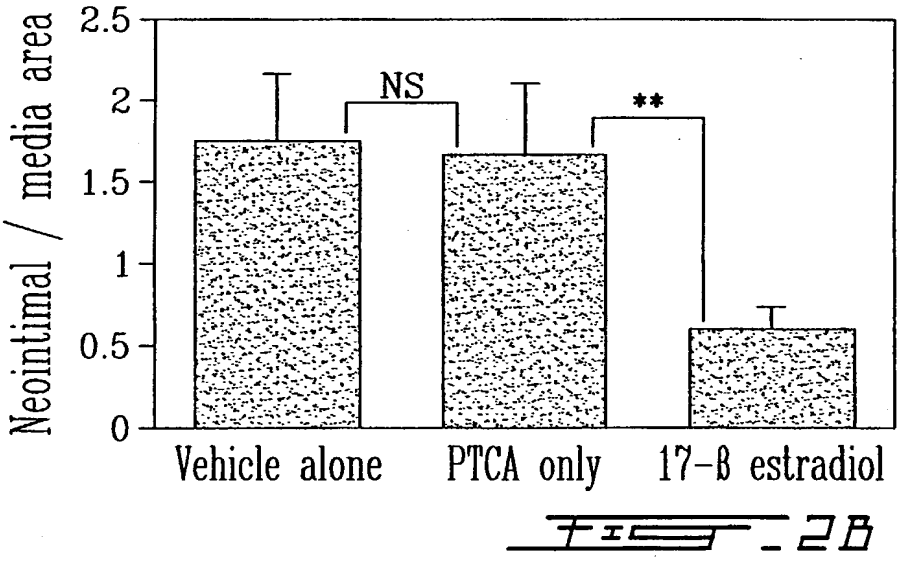

Neointimal / media area
2.5
2
1.5
1
0.5
0
NS
**
Vehicle alone
PTCA only
17-ß estradiol

Fig 2B

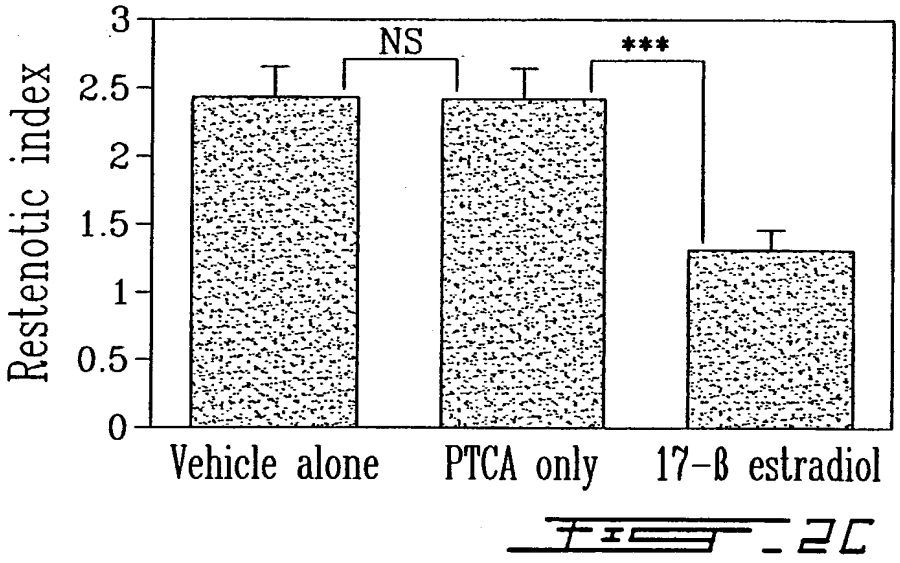

Restenotic index
3
2.5
2
1.5
1
0.5
0
NS
***
Vehicle alone
PTCA only
17-ß estradiol

Fig 2C

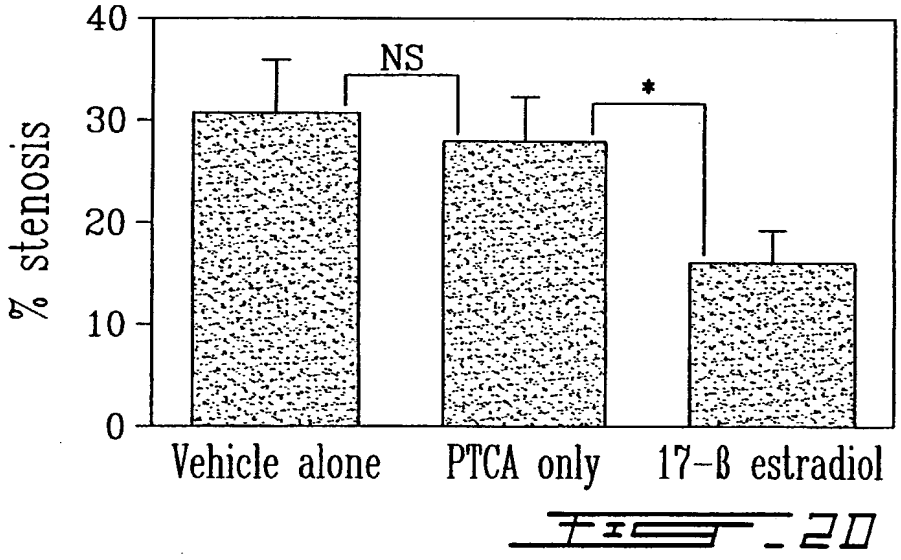

% stenosis
40
30
20
10
0
NS
*
Vehicle alone
PTCA only
17-ß estradiol

Fig 2D

FIG 3A FIG 3B FIG 3C

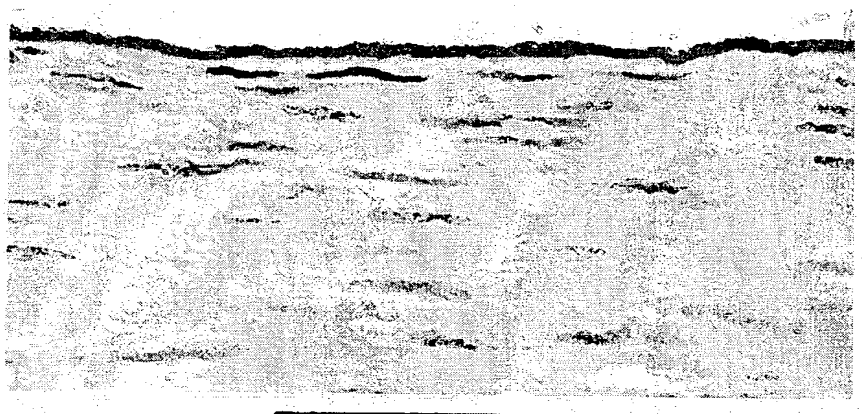


Fig 4A

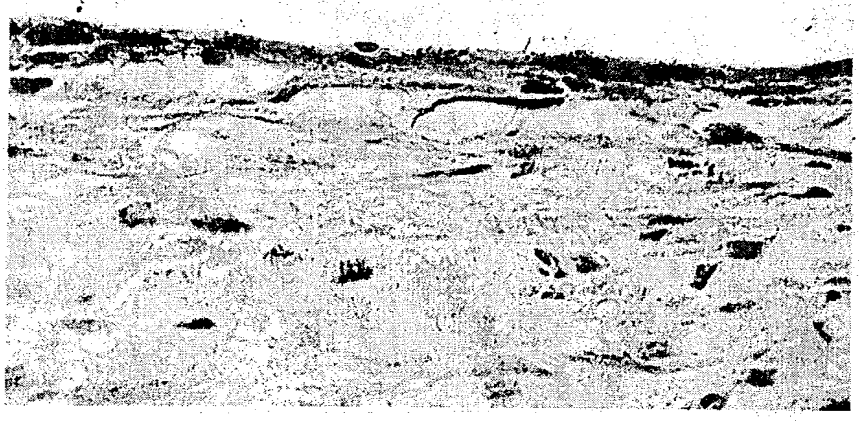


Fig 4B

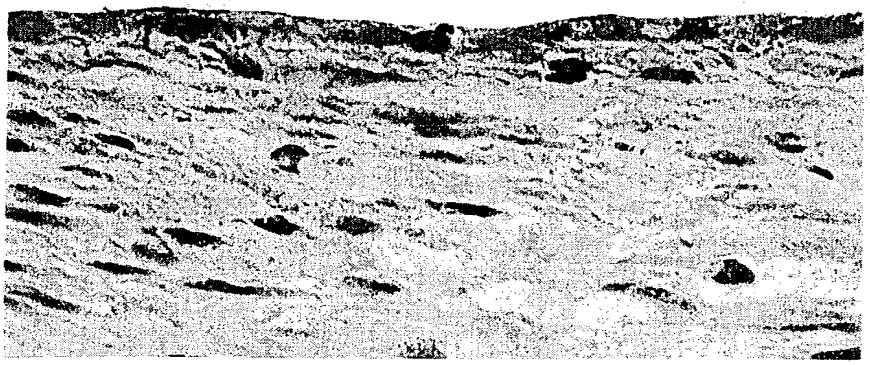


Fig 4C

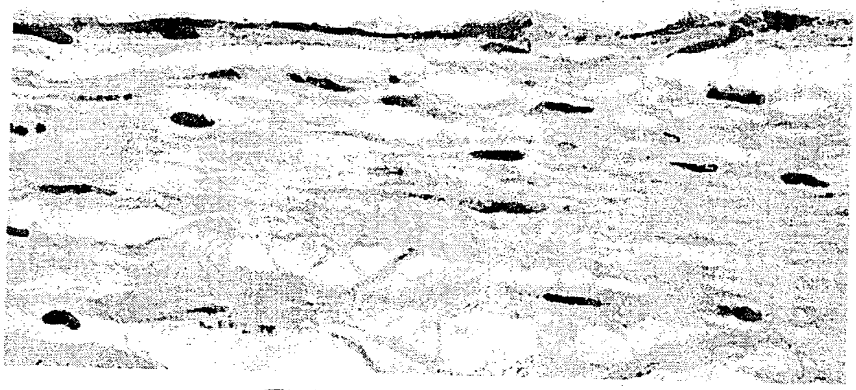


Fig 5A

Fig 5B

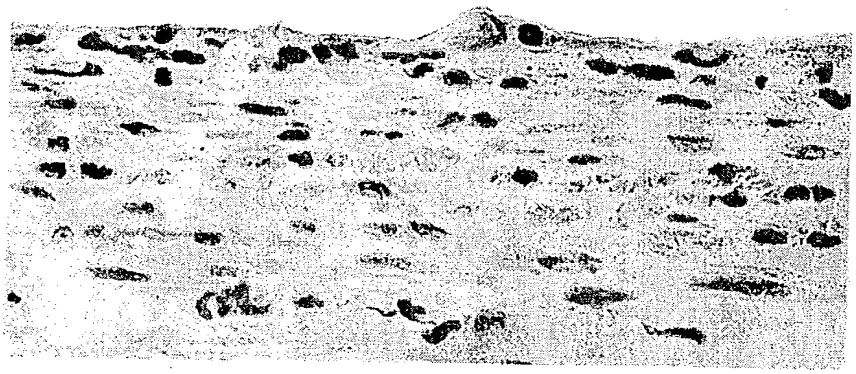


Fig 5C

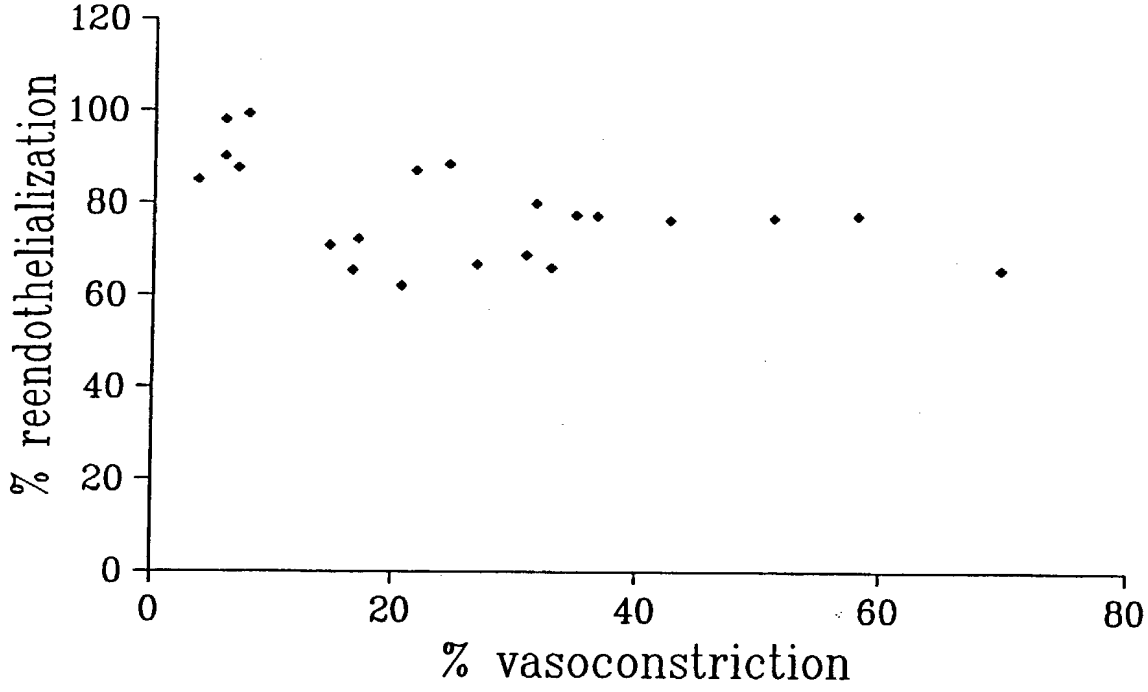

% reendothelialization
120
100
80
60
40
20
0
0
20
40
60
80
% vasoconstriction

Fig 6A

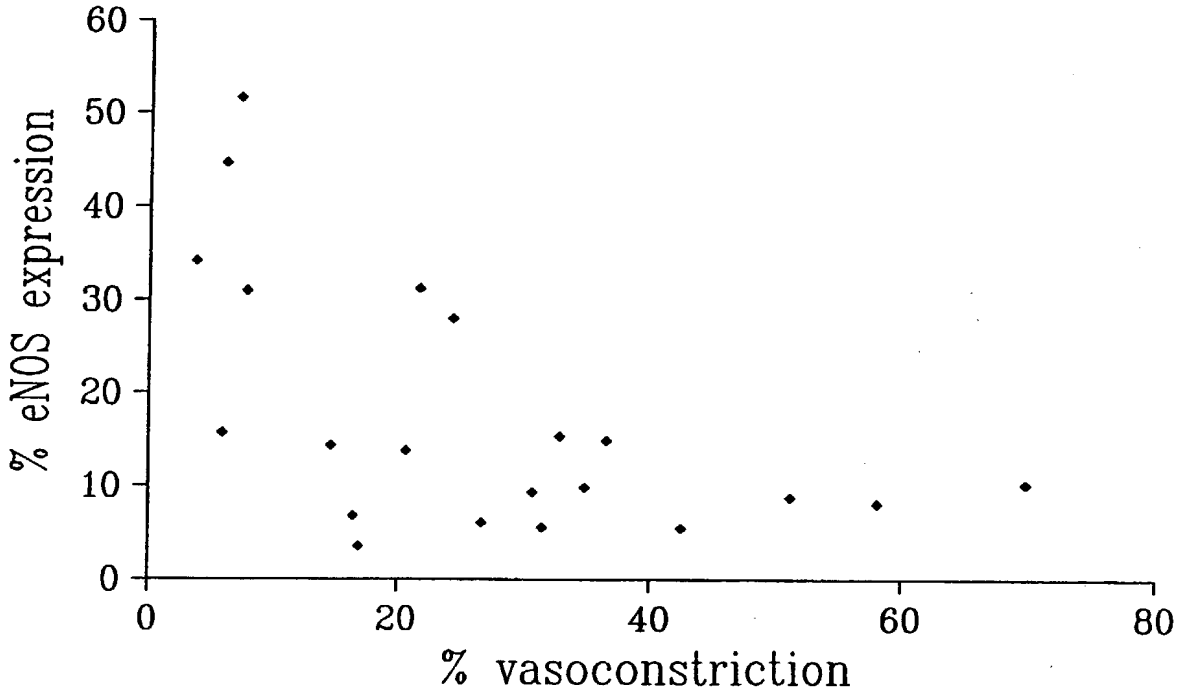

% eNOS expression
60
50
40
30
20
10
0
0
20
40
60
80
% vasoconstriction

Fig 6B

US

COMBINED DECLARATION FOR PATENT APPLICATION AND POWER OF ATTORNEY (Includes Reference to PCT International Applications)	ATTORNEY DOCKET NUMBER 410718.90395

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name.

I believe that I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

LOCAL DELIVERY OF 17-BETA ESTRADIOL FOR PREVENTING VASCULAR INTIMA HYPERPLASIA AND FOR IMPROVING VASCULAR ENDOTHELIUM FUNCTION AFTER VASCULAR INJURY

the specification of which (check only one item below):

[] is attached hereto.

[] was filed as U.S. Patent Application Serial Number__
on ___,
as amended on ____ (if applicable).

[X]was filed as a PCT international application number PCT/CA00/01132 on 21 Sept 2000
as amended under PCT Article 19 on 11/19/01 (if applicable).

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to the examination of this application in accordance with Title 37, Code of Federal Regulations §1.56(a).

I hereby claim foreign priority benefits under Title 35, United States Code, §119 of any foreign application(s) for patent or inventor's certificate or any PCT international application(s) designating at least one country other than the United States of America listed below and have also identified below any foreign application(s) for patent or inventor's certificate or any PCT international application(s) designating at least one country other than the United States of America filed by me on the same subject matter having a filing date before that of the applications for which priority is claimed:

PRIOR FOREIGN PATENT APPLICATION(S) AND ANY PRIORITY CLAIMED UNDER 35 U.S.C. §119:

COUNTRY (If PCT Indicate PCT)	APPLICATION NUMBER	DATE OF FILING (Day, Month, Year)	PRIORITY CLAIMED UNDER 35 USC 119
PCT	PCT/CA00/01132	21 Sept 2000	[X] YES [ ] NO
CA	2,282,982	21 Sept 1999	[X] YES [ ] NO
CA	2,300,246	09 March 2000	[X] YES [ ] NO
			[ ] YES [ ] NO

US

COMBINED DECLARATION FOR PATENT APPLICATION AND POWER OF ATTORNEY (Includes Reference to PCT International Applications)	ATTORNEY DOCKET NUMBER 410178.90395

I hereby claim the benefit under Title 35, United States Code, §120 of any United States application(s) or PCT international application(s) designating the United States of America that is/are listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in that/those prior application(s) in the manner provided by the first paragraph of Title 35, United States Code, §112, I acknowledge the duty to disclose material information as defined in Title 37, Code of Federal Regulations §1.56(a) which occurred between the filing date of the prior application(s) and the national or PCT international filing date of this application.

PRIOR U.S. APPLICATIONS OR PCT INTERNATIONAL APPLICATIONS DESIGNATING THE U.S. FOR BENEFIT UNDER 35 U.S.C. 120:

U.S. APPLICATIONS		STATUS (Check One)		
U.S. APPLICATION NUMBER	U.S. FILING DATE	PATENTED	ABANDONED	PENDING

PCT APPLICATIONS DESIGNATING THE U.S.					
PCT APPLICATION NUMBER	PCT FILING DATE	U.S. SERIAL NUMBERS			

POWER OF ATTORNEY: As a named inventor, I hereby appoint the following attorneys and/or agents to prosecute this application and transact all business in the U.S. Patent and Trademark Office connected therewith (List names and registration numbers):

Send Correspondence to:	Direct Telephone Calls to:
Jean C. Baker Quarles & Brady LLP 411 East Wisconsin Ave. Suite 2550 Milwaukee, WI 53202-4497	(414) 277-5000

201	FULL NAME OF INVENTOR	FAMILY NAME CHANDRASEKAR	FIRST GIVEN NAME Baskaran	SECOND GIVEN NAME
	RESIDENCE & CITIZENSHIP	CITY Dr. Radhakrishnan Nagar	STATE OR COUNTRY Chennai /NX	COUNTRY OF CITIZENSHIP India
	POST OFFICE ADDRESS	POST OFFICE ADDRESS 2, Justice Ramanujam Road	CITY Dr. Radhakrishnan Nagar, Chennai	STATE & ZIP CODE/COUNTRY India 600 041
202	FULL NAME OF INVENTOR	FAMILY NAME TANGUAY	FIRST GIVEN NAME Jean-Francois	SECOND GIVEN NAME
	RESIDENCE & CITIZENSHIP	CITY Montreal	STATE OR COUNTRY Quebec CAX	COUNTRY OF CITIZENSHIP Canada
	POST OFFICE ADDRESS	POST OFFICE ADDRESS C.O. 323, Succ. Mont-Royal	CITY Montreal, Quebec	STATE & ZIP CODE/COUNTRY Canada H3P 3C5
203	FULL NAME OF INVENTOR	FAMILY NAME	FIRST GIVEN NAME	SECOND GIVEN NAME
	RESIDENCE & CITIZENSHIP	CITY	STATE OR COUNTRY	COUNTRY OF CITIZENSHIP
	POST OFFICE ADDRESS	POST OFFICE ADDRESS	CITY	STATE & ZIP CODE/COUNTRY

1-00

2-00

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

SIGNATURE OF INVENTOR 201	SIGNATURE OF INVENTOR 202	SIGNATURE OF INVENTOR 203
DATE 29/5/2002	DATE 29/5/2002	DATE

United States Patent & Trademark Office

Office of Initial Patent Examination -- Scanning Division

Application deficiencies found during scanning:

☐ Page(s)_____of_____ were not present for scanning. (Document title)

☐ Page(s)_____of_____were not present for scanning. (Document title)

☐ ***Scanned copy is best available.*** some drawings are dark